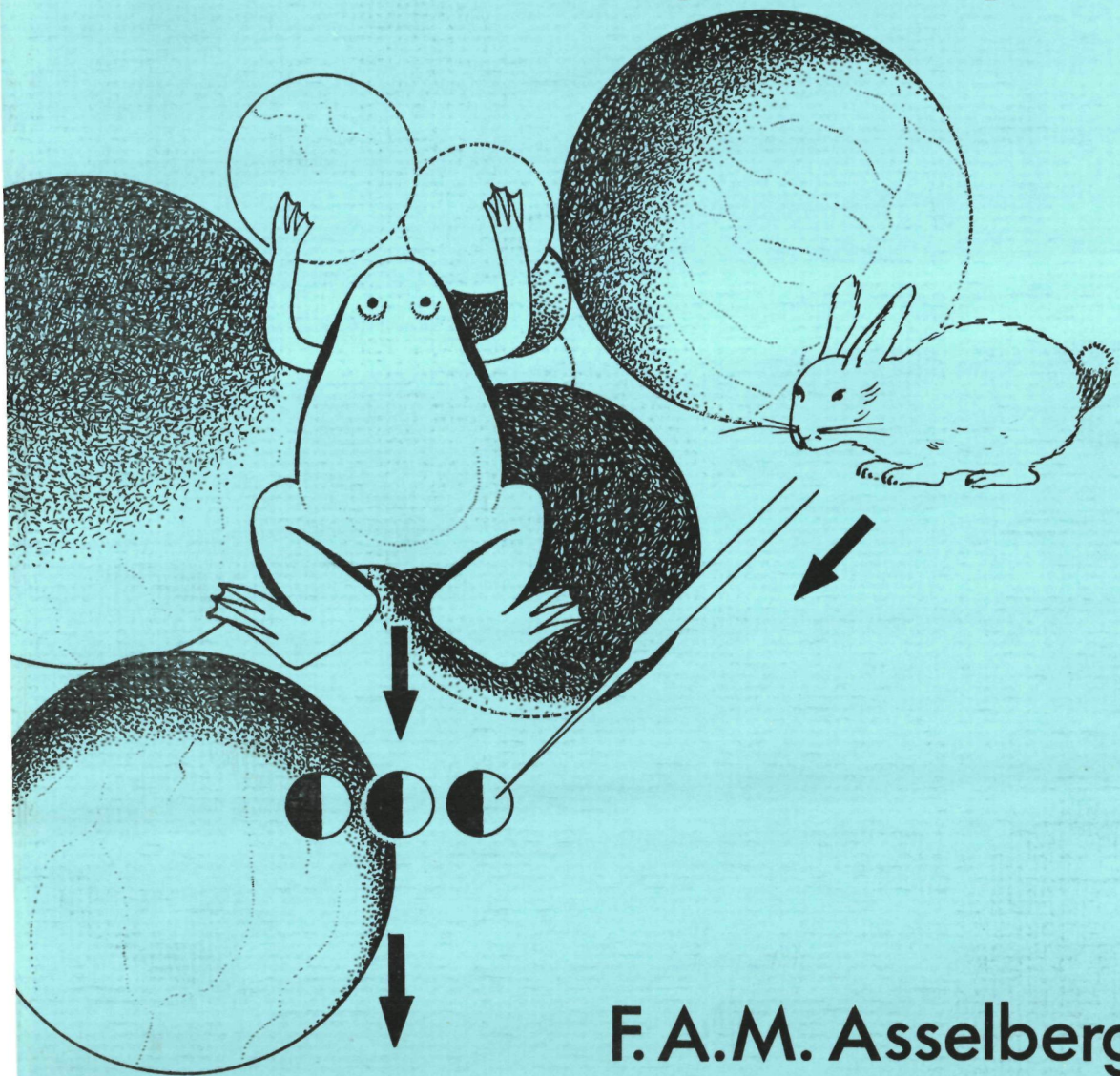
2403

# Protein Synthesis on Heterologous messenger RNA in Xenopus oocytes



F. A. M. Asselbergs

# PROTEIN SYNTHESIS ON HETEROLOGOUS MESSENGER RNA IN XENOPUS OOCYTES

PROMOTOR:

PROF. DR. H. BLOEMENDAL

CO - REFERENT:

DR. W.J. VAN VENROOIJ

# PROTEIN SYNTHESIS ON HETEROLOGOUS MESSENGER RNA IN XENOPUS OOCYTES

**PROEFSCHRIFT**

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. P.G.A.B. WIJDEVELD
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 8 NOVEMBER
DES NAMIDDAGS OM 2 UUR PRECIES

door

FREDERICUS ALPHONSUS MARIA ASSELBERGS
geboren te Bergen op Zoom

1979

Druk: Offsetdrukkerij Faculteit der Wiskunde en Natuurwetenschappen
Nijmegen

Motto: REM ACU TETIGI (Plautus)

AAN MIJN VADER EN MIJN MOEDER

Bij het uitvoeren van de experimenten, die tesamen dit proefschrift vormen, heb ik veel steun ondervonden van talloze medewerkers van de Katholieke Universiteit, die min of meer verborgen blijven achter de schermen van de universitaire organisatie. Niets van dit alles zou tot stand zijn gekomen zonder de goede verzorging van de dieren, met name die van de klauwpadden, zonder de assistentie van de medewerkers van het Centraal Dierenlaboratorium bij de isolatie van konijnenbloed en bij de vele immunisaties, zonder de hulp van de student-assistenten, die de lenspolysomen isoleerden, en zonder de technische bijstand van de instrumentmakerijen van de faculteiten Geneeskunde en Wiskunde en Natuurwetenschappen. De afdelingen Medische Illustratie en Medische Fotografie verzorgden op een voortreffelijke manier de vele tekeningen en foto's. De afdeling Genetica dank ik voor het gebruik van de apparatuur voor het maken van de injectiecapillairen.

Mijn bijzondere dank gaat uit naar Marion Koopmans voor het analyseren van de oocyten van de in totaal bijna 50 padden. Haar praktisch organisatietalent maakte het voor mij mogelijk om mijn aandacht te verdelen over een groot aantal parallel lopende experimenten. Ook Annemarie Selten - Versteegen wil ik op deze plaats niet vergeten. De hulp van Frans Ramaekers, Wilbert Peeters en Eline Meulenberg, gegeven in het kader van hun doctoraal-stage biochemie, heeft veel bijgedragen tot de resultaten van dit onderzoek. Cobi van Breda dank ik voor het maken van de coupes van de *Xenopus* oocyten. De leerzame en stimulerende samenwerking met mijn collega's van de afdeling Biochemie, in het bijzonder met Martin Salden, heb ik zeer op prijs gesteld.

CONTENTS

# CHAPTER I

# INTRODUCTION

## INJECTION OF PURIFIED MACROMOLECULES INTO AMPHIBIAN OOCYTES

## Introduction: Injection of purified macromolecules into amphibian oocytes: A compromise

This thesis deals primarily with experiments in which macromolecules isolated from animal and plant cells are injected into the oocytes of the African clawed frog, Xenopus laevis. The large size of these cells provides the molecular biologist with the possibility to introduce precise amounts of macromolecules of biological interest into a living cell and makes it possible to (re-)isolate macromolecules from an injected cell. This type of non-genetic recombination experiment is in a sense a compromise, since it is at most a close alternative to what molecular biologists really want to do: to study the function of purified macromolecular cell components by reconstituting them into a living cell. However, this is a dream far beyond our present possibilities, which only allow us partial reconstitution experiments in vitro. In so-called cell-free systems we can manipulate the individual components very easily. Unfortunately, many macromolecules cannot display all aspects of their function in vitro due to the incompleteness of cell-free systems. Alternatively we can use the powerful isolation techniques of biochemistry to study the pathways of macromolecules in vivo as they are influenced by (manipulatable) factors outside the cells. The microinjection approach may also be considered a compromise in that it is a combination of the in vitro and the in vivo experiment: being able to manipulate the intracellular components of a cell under the conditions of the in vivo experiment.

In addition to a series of experiments of the author on microinjection of mRNA in Xenopus oocytes, this thesis contains also a

description of the technical aspects of microinjection and of the biological features of the Xenopus oocyte as well as an up to date review on microinjection of mRNA. The author hopes that the collection of articles presented here will provide useful background information for future investigators planning experiments with oocytes and that it will further enhance the popularity of this versatile experimental system. The rest of this introductory chapter contains a brief summary of the contents of the thesis.

Chapter II discusses the problems and possibilities of microinjection into Xenopus oocytes and compares them with other microinjection systems. Apart from their size, amphibian eggs and oocytes have several advantages as compared to other micro-injection systems. Their unique position in the development of the organism has made the oocytes the subject of many experiments on the molecular biology of early development. This, in turn, has resulted in an abundance of data on these cells, of which a concise review is given with emphasis on the implications for microinjection experiments.

The oocyte system has been remarkably popular, especially in the field of translation of eukaryotic mRNA. In fact, it was by using oocytes that it was first demonstrated that a purified mRNA from one kind of eukaryotic cell (9S globin mRNA from rabbit reticulocytes) is acceptable to the translational apparatus of another cell type (the oocyte) (Lane, Marbaix and Gurdon, 1971). This type of experiment has since then been repeated with many other mRNA species, listed in section III-1. This section contains a review of how microinjection of mRNA

into Xenopus oocytes has contributed to our understanding of the post-transcriptional regulation of protein synthesis. All available evidence indicates that not the concentration of mRNA itself, but other factors limit and probably regulate protein synthesis in oocytes. A discussion model of the possible compartmentalization of the cellular mRNA pool is used to describe the functional characteristics of the regulatory factors. One type of experiment providing information about the functional properties of the translational apparatus is to analyze the competition between two or more mRNA species. This requires a method to measure simultaneously the rate of translation of several mRNA species. In section III-2 (Asselbergs, van Venrooij and Bloemendal, 1978a) it is demonstrated that by quantitative immunoprecipitation it is possible to monitor simultaneously the synthesis of several species of lens crystallins in oocytes microinjected with calf lens RNAs. The translation of rabbit globin mRNA can be assayed by gel electrophoresis of the proteins synthesized by the oocyte. These techniques have been used to compare separately the translation of globin mRNA with that of one purified lens mRNA species, $\alpha A_2$-crystallin mRNA, in oocytes. This methodology has also been applied to mRNA competition experiments in oocytes (section III-3: Asselbergs, van Venrooij and Bloemendal, 1979) and in an mRNA dependent reticulocyte lysate (Asselbergs, Meulenberg, van Venrooij and Bloemendal, 1979). The constraints on mRNA translation in oocytes are shown to be entirely different from those <u>in vitro</u>. At high mRNA concentration certain mRNA species are preferentially translated by the cell-free system. This shift in the selectivity of the translational apparatus in response to an increased concentration of particular mRNA species is only compatible with a model in which different factors

limit mRNA translation of different (groups) of mRNA species in vitro. In oocytes at high mRNA concentration, no shift in the selectivity for the injected mRNA, is observed although at all concentrations some injected mRNA species are translated with higher efficiency than other. Messenger RNAs already being translated, such as the oocyte endogenous mRNAs, are less sensitive to competitive inhibition by injected mRNAs. As the injected mRNAs gradually become incorporated in the translational machinery of the oocyte, they acquire the same status in respect to competitive inhibition as the oocyte endogenous mRNAs (section III-3: Asselbergs, van Venrooij and Bloemendal, 1979). Attempts to inhibit translation of mRNA in oocytes in a competitive manner using only a small part of the mRNA molecule (cap-analogues are equivalent to the 5' end of eukaryotic mRNA) failed for unknown reasons (section III-4: Asselbergs, Peters, van Venrooij, and Bloemendal, 1978a), although strong inhibition was observed in the reticulocyte lysate (Asselbergs, Peters, van Venrooij and Bloemendal, 1978 b,c,d).

Part of the popularity of the Xenopus oocyte for the translation of all kinds of mRNA is related to its capacity to modify many of the newly synthesized polypeptide chains after translation just as the cell from which the mRNA originated. The different types of post-synthetic modification in oocytes, microinjected with heterologous mRNA, are reviewed in section IV-1. One particularly interesting type of post-synthetic 'modification' is the assembly of two or more polypeptide chains into multimeric proteins. The α-crystallin molecule is an example of such a multimeric protein, being composed of about 40 αA- and αB- subunits. Correct assembly of α-crystallin is observed in oocytes, microinjected with lens mRNA, as is demonstrated in

section IV-2 (Asselbergs, Koopmans, van Venrooij and Bloemendal, 1978). Such an assembly does not oocur in a cell-free system probably because the concentration of newly synthesized α-crystallin subunits synthesized in vitro is not high enough.

The last section of this thesis is devoted to the translation of viral RNA in oocytes. Apart from the translation of viral RNA and its regulation, the post-synthetic modification of viral translation products and the possible role therein of viral gene expression (also discussed in section IV-1), this chapter covers a feature so far exclusively found in RNAs of viral origin. Some viral RNAs contain the genetic information for multiple translation products. However, when these RNAs are translated only the one encoded by the 5' part of the molecule is synthesized. The information which is silent in the large RNAs is expressed in shorter RNA molecules, subgenomic mRNAs, which differ from the viral (genomic) RNAs in that a part of the large RNA is missing in the shorter type. Presumably, subgenomic mRNAs are generated by scission or 'splicing' (i.e. removal of an internal piece) of a large precursor RNA of the size of the genomic RNA. After an introduction (section V-1), it is shown that micro-injected genomic RNAs of two oncogenic RNA viruses, avian myeloblastosis virus (section V-2: Salden, Asselbergs and Bloemendal, 1976) and Rauscher murine leukemia virus (section V-3: Asselbergs, Salden and Bloemendal, 1979), are translated in oocytes as such. No translation products of subgenomic mRNAs could be detected.

References

1. Asselbergs, F.A.M., Koopmans, M., van Venrooij and Bloemendal, H. (1978) Post-translational assembly of lens α-crystallin in the reticulocyte lysate and in Xenopus laevis oocytes. Eur. J. Biochem. 91, 65-72 (section IV-2).

2. Asselbergs, F.A.M., Meulenberg, P.M.M., van Venrooij, W.J. and Bloemendal, H. (1979) Unequal competition between messenger RNAs in a messenger dependent reticulocyte lysate, Submitted for publication.

3. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij, W.J. and Bloemendal, H. (1978a) Cap-analogues do not inhibit mRNA translation in Xenopus oocytes. FEBS Lett. 94, 195-198 (section III-4).

4. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij, W.J. and Bloemendal, H. (1978b) Inhibition of translation of lens mRNAs in a messenger dependent reticulocyte lysate by cap-analogues. Biochim. Biophys. Acta. 520, 577-587.

5. Asselbergs, F.A.M., Peters, W., van Venrooij, W.J. and Bloemendal, H. (1978c) Diminished sensitivity of re-initiation of translation to inhibition by cap-analogues in reticulocyte lysates. Eur. J. Biochem. 88, 483-488.

6. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij, W.J. and Bloemendal, H. (1978d) The effect of the messenger RNA concentration on the competitive inhibition of translation by cap-analogues. Mol. Biol. Rep. 4, 177-180.

7. Asselbergs, F.A.M., Salden, M.H.L. and Bloemendal, H. (1979) Kinetics of synthesis and processing of the precursor polypeptides of murine leukemia virus in frog oocytes following microinjection of viral RNA. Submitted for publication (section V-3).

8. Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H. (1978a) Synthesis of lens crystallins in Xenopus oocytes as determined by quantitative immunoprecipitation. Eur. J. Biochem. 87, 517-524 (section III-2).

9. Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H. (1979) Messenger RNA competition in living Xenopus oocytes. Eur. J. Biochem. 94, 249-254 (section III-3).

10. Lane, C.D., Marbaix, G. and Gurdon, J.B. (1971) Rabbit haemoglobin synthesis in frog cells: The translation of reticulocyte of 9S RNA in frog oocytes. J. Mol. Biol. 61, 73-91.

11. Salden, M.H.L., Asselbergs, F.A.M. and Bloemendal, H. (1976) Translation of oncogenic virus RNA in Xenopus laevis oocytes. Nature 259, 696-699. (section V-2).

# CHAPTER II

## USING A LIVING CELL AS TEST TUBE

How to introduce a macromolecular into a living cell?

Why use amphibian eggs and oocytes?

The Xenopus ovary and the ovarian follicle.

Stages of oogenesis in Xenopus laevis.

Are stage VI oocytes dormant cells?

Micro-injection of oocytes.

Incubation and labeling of oocytes.

Micro-injection of Xenopus eggs.

Analysis of the proteins synthesized in oocytes.

Micro-injection into the oocyte nucleus.

CHAPTER II

## USING THE LIVING CELL AS TEST-TUBE

Much of the success of molecular biology has stemmed from the development of methods for purifying macromolecules and from studying their behavior *in vitro*. However a persistent question in the interpretation of such experiments is whether results obtained under *in vitro* conditions can be extrapolated in order to understand *in vivo* phenomena. If purified macromolecules are smuggled into the subcellular machinery, one may circumvent this problem. This approach has, for example, been used with success in studying the translation of messenger RNAs (chapters III and IV). Not only is the 'contraband' mRNA readily accepted by the cellular ribosomes, but the resulting translation product is correctly processed by the cellular machinery for post-synthetic modification of polypeptides (chapters IV and V). This introduction deals primarily with the technical problems and possibilities of one type of such experiments, namely the injection of nucleic acids into amphibian eggs and oocytes.

### How to introduce a macromolecule into a living cell?

One of the easiest ways of following the pathway of one particular type of macromolecule inside a living cell is to infect the cell with a virus. The outer layer of the coat of the virus will attach to the cell membrane, whereupon the content of the viral coat, the nucleic acid plus some viral proteins, is efficiently taken up by the cell. Penetration of infectious nucleic acids without the help of the viral coat proteins is possible. However, it occurs with an efficiency several orders of

magnitude lower. Unfortunately most viruses change the metabolism of macromolecules of the cell itself and are thus not ideal tracer molecules, which should participate in, but not alter the cellular machinery.

Spontaneous uptake of DNA-fragments followed by transformation (Avery, MacLeod and McCarthy, 1944) has also been observed in eukaryotic cells, for example with isolated thymidine kinase genes (Wigler, Pellicer, Silverstein and Axel, 1978). However successful with DNA, this approach is hard to use with non-replicative macromolecules such as RNAs and proteins. Limited success has only been obtained by introducing messenger RNAs in the lumen of the (oestrous) mouse uterus. Apparently some cells of the uterine wall take up the mRNA (coding for chicken avidin (Tuohimaa and Soederling, 1976) or for albumin of various species (Yang and Niu, 1977) and translate it correctly. Although this kind of experiment allows some qualitative conclusions, it is a major disadvantage that the uptake of nucleic acids (or other macromolecules) cannot be quantified.

Recently a new and very promising method of introducing large quantities of macromolecules into tissue culture cells has been developed as a 'spin off' from cell fusion experiments. It is referred to as 'ultramicroinjection' (Loyter, Zakai and Kulka, 1975) or 'chemically facilitated injection' (Kriegler and Livingston, 1977). This method consists in enclosing the macromolecules in erythrocyte ghosts, which are then fused with the target cell with the help of inactivated Sendai virus (Loyter et al., 1975) or polyethylene glycol (Kriegler and Livingston, 1977). Enclosing the macromolecules to be injected into liposomes (Dimitriados, 1978; Ostro, Giacomo, Lavelle, Paxton and Dray, 1978) may represent another improvement since then no fusogenic substance is

needed. It is possible, that this method may be developed further into a tool for quantitative experiments. However, as yet it is not very efficient. One author reported that only 0.2% of the starting material was actually transferred into the tissue culture cells (Loyter et al., 1975). At the present moment, therefore, the most reliable way of introducing informational macromolecules into living cells is micro-injection.

Why use amphibian eggs and oocytes?

The choice of recipient cell depends of course on the type of experiments one has in mind. In our case the cell of choice should allow the injection of quantifiable amounts of messenger RNA and it should synthesize enough of the translation product to allow direct (quantitative) biochemical analysis. Technically it is now possible to detect translation of messenger RNAs injected into tissue culture cells (Graessmann and Graessmann, 1971, 1976; Stacey and Allfrey, 1976; Stacey, Allfrey and Hanafusa, 1977). However, because of the small size of such cells (10 - 20 $\mu m^3$), it is as yet difficult to control the injection volume with sufficient accuracy. Also a major limitation is that translation products cannot be quantitated using standard biochemical procedures, but that instead assays which work at the single cell level e.g. immunofluorescence or indirect methods, such as looking for virus yield, must be used. These restrictions can be circumvented by using larger cell types, such as protozoal cells (100 $\mu m^3$) (Knowles, Lipps and Nook, 1978), (fragments of) giant algae cells (up to 4 cm long) (Cairns, Gschwender, Primke, Yamakawa, Traub and Schweiger, 1978) or amphibian eggs and oocytes (1-2 $mm^3$). Especially the eggs and oocytes of the South African clawed frog, Xenopus laevis, have frequently been used

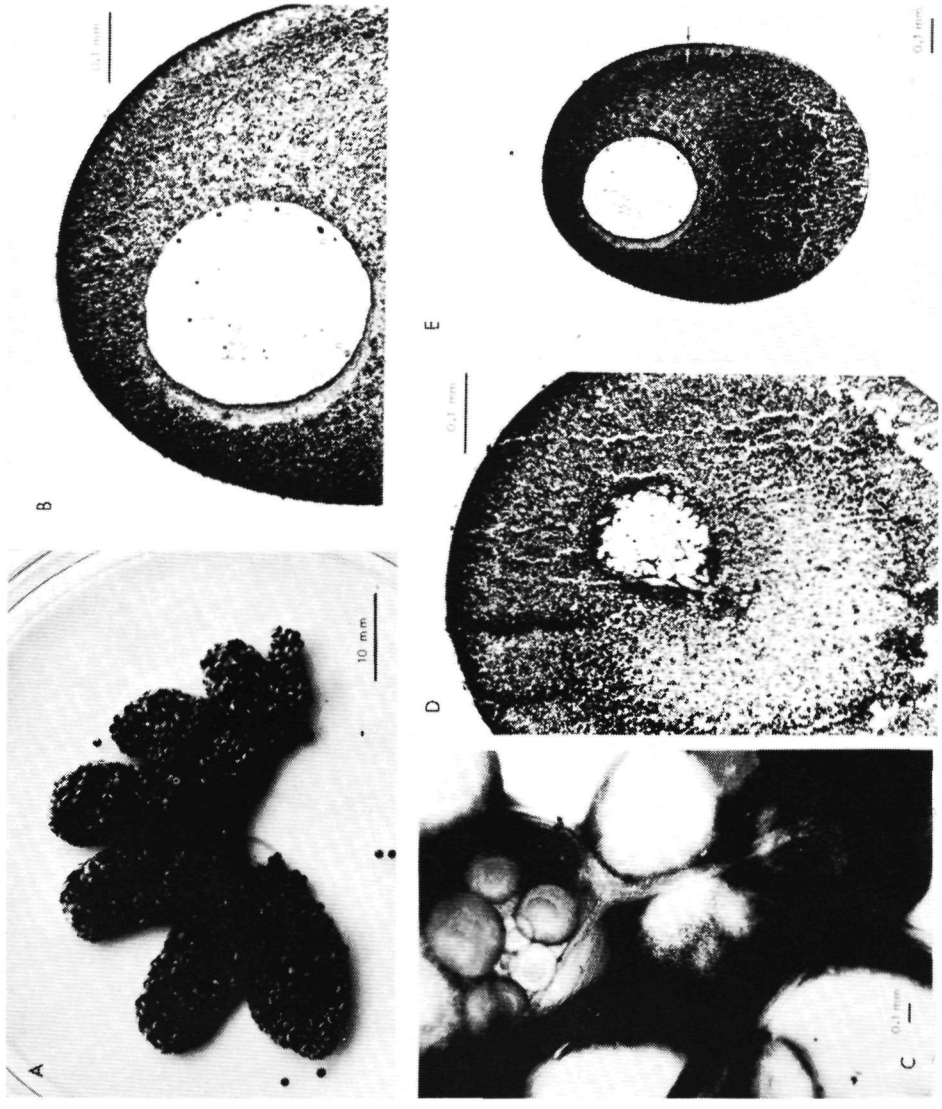

A
10 mm
B
0.1 mm
C
0.1 mm
D
0.1 mm
E
0.1 mm

fig. 1: Oocyte morphology

(A) Part (about 10%) of a Xenopus ovary, showing the lobal structure of the organ. (B) as (E), but at higher magnification. (C) Surface view of the ovary with previtellogenic oocytes (with the nucleus visible inside it), oocytes at various stages of vitellogenesis and blood vessels in the theca (layer of connective tissue which surrounds the oocytes). (D) Cross-section of stage V oocyte (at the end of vitellogenesis), showing oocyte nucleus (germinal vesicle) and yolk granules. Oocytes were fixed in 4% formaldehyde and stained with haematoxyline/eosin. (E) Cross-section, which is oriented in such a way, that the animal pole is at the top just above the nucleus, in which several nucleoli can be seen. Arrows indicate the pigment layer of the animal hemisphere of the oocyte.

in microinjection studies, since oogenesis and early embryological development in this species have been characterized in great detail.

The Xenopus ovary and the ovarian follicle

The ovary is a thin sac-like multilobed organ (fig. 1A), covered at the inner and the outer side with a squamous epithelium. The oocytes develope in the connective tissue stroma between the two epithelia. The large oocytes bulge out towards the inner side of the ovary. The surface of the ovary at the side of the body cavity is smooth. Directly under the surface large bloodvessels can be seen (fig. 1C). An excellent description of the Xenopus ovary with the developing oocytes and their investing follicular tissues was recently published by Dumont and Brummet (1978). We will, therefore, restrict ourselves to those features important for the in vitro culture of oocytes.

Subsequent to stage I (below) the oocyte is surrounded by the acellular vitelline envelope (a), peripheral to which lies a single layer of follicle cells (b), itself encased in a connective tissue layer, the theca (c), which is vascularized except at the ovulation site. From this point, the mature oocyte will be released from the surrounding follicle into the body cavity. Since the large oocytes protrude from the inner side of the ovarian wall, most of the theca of the oocyte is covered by the inner ovarian epithelium (d). Thus, four layers (a-d) may surround the oocytes when they are manually removed from the ovary. Extending from the margins and the basal surface of the follicle cells are long processes, called macrovilli, which penetrate the vitelline envelope and contact the developing oocyte making specialized cell junctions. The layer of follicle cells around the oocyte is not entirely contiguous. The oocyte also appears to be linked directly

to the bloodvessels in the theca via a system of channels. It may be that these intimate contacts of the oocyte with the surrounding tissue are also important under in vitro conditions, since manually defolliculated oocytes are less suitable for long term culture especially in non-nutrient media (J. Ghysdael, personal communication). Xenopus oocytes are reported to be grouped in clusters of 16 oocytes, which develope synchronously (Coggins, 1973). Perhaps this is the reason why for long term culture clumps of oocytes are sometimes preferred over single oocytes.

Stages of oogenesis in Xenopus laevis

During oogenesis the growing oocyte gradually acquires all the materials needed to sustain the growth and development of the future embryo (table 1) (Davidson, 1976; Dumont, 1972). In Xenopus oogenesis is a continuous, asynchronous process and oocytes in all stages of development are present in the ovary at all times during adult life (fig. 1C) [The only exceptions being females recently induced to lay eggs (by the administration of hormone (Dumont, 1972) or, indirectly, by environmental factors in their natural habitat) and, in our experience, animals, that recently have suffered unfavorable aquarium conditions. The large oocytes then become atretic and are resorbed by the animal. In such frogs only small immature oocytes are found.] Dumont (1972) has divided oogenesis in Xenopus into six stages, each with a characteristic morphology (table 1). Accumulation of the various components of the subcellular machinery for later use in the embryo occurs at different stages of development of the oocyte. For example, the accumulation of 5S ribosomal RNA and tRNA (Denis, 1977), as well as accumulation of a

Table 1 STAGES OF OOGENESIS IN XENOPUS LAEVIS (DAUDIN)

| STAGE[1)] | SIZE (nm) | MORPHOLOGY | BIOCHEMICAL CHARACTERISTICS |
|---|---|---|---|
| I | 50 to 300 | transparent cytoplasm nucleus clearly visible | previtellogenic, beginning of amplification of rDNA, maximal synthesis and accumulation of 5S rRNA and tRNA (Denis, 1977) and mRNAs with long poly(A) segments (Rosbash and Ford, 1974; Cabada et. al., 1977). |
| II | 300 to 450 | cytoplasm translucent early, white and opaque later | early vitellogenic, small yolk platelets appear in the periphery beginning of the accumulation of 18S and 28S rRNA (appearance of the lampbrush chromosomes), accumulation of mitochondrial DNA (Webb and Smith, 1977). Start of accumulation of mRNAs with short poly(A) segments (Cabada et. al., 1977). |
| III | 450 to 600 | pigment forms, light brown early, uniform blackish-brown later | mid-vitellogenic, yolk platelets become larger but not yet crystalline, maximal accumulation of 18S and 28S rRNA (maximal extension of the lampbrush chromosomes) |
| IV | 600 to 1000 | animal and vegetal hemispheres become differentiated | mid-vitellogenic, maximal vitellogenesis. Lampbrush chromosomes start of condense, end of accumulation of mitochondrial DNA. |
| V | 1000 to 1200 | hemispheres clearly delineated, animal hemisphere appears light brown | late vitellogenic, yolk accumula- decreases, nucleus becomes displaced towards the animal pole. |
| VI | 1200 to 1300 | unpigmented equatorial band, animal pole becomes blackish-brown again | post-vitellogenic, polarization of large crystalline yolk platelets in the vegetal hemisphere. End of accumulation of mRNA with short poly(A). (Cabada et.al.,1977) |
| maturation | | release from the follicle, formation of the jelly coat, oocyte becomes an egg. | disappearance of the nuclear membrane (= germinal vesicle breakdown), begin of meiosis, activation of the apparatus for DNA synthesis. |

1) according to Dumont (1972).

population of messenger RNAs with long poly(A) segments (Rosbash and Ford, 1974; Cabada, Darnbrough, Ford and Turner, 1977) preceeds the accumulation of the 18 and 28S ribosomal RNAs (Davidson, 1976). About half of the oocyte 5S rRNA, which is slightly different in sequence from somatic 5S rRNA (Denis, 1977) is initially stored together with tRNA and some (non-ribosomal) proteins in the form of 42S particles. Recently it was found that the other half of the 5S RNA is stockpiled in 7S particles together with a 45,000-dalton protein. (Picard and Wegnez, 1979). Assembly of 5S rRNA into ribosomes awaits embryogenesis. Similarly, there is a lag-period between the amplification of the genes encoding the 18 and 28S ribosomal RNAs and their transcription (Davidson, 1976), an event which takes place in the many nucleoli of the germinal vesicle as the oocyte nucleus is called. (fig. 1 B,E). The most conspicuous process during oogenesis, however, is vitellogenesis, the deposition of large amounts of yolk in the oocyte. The two yolk proteins, lipovitellin (three polypeptide chains of 105, 35 and 32 kDalton (Ohlendorf, 1977)) and phosvitin (17-19 kDalton), are synthesized in the liver as a precursor polypeptide, vitellogenin (Tata, 1976). This precursor circulates in the blood as a serum protein and is taken up by the oocytes by pinocytosis (Jared and Wallace, 1969). In the oocyte vitellogenin is cleaved and the lipovitellin and phosvitin are assembled to form the crystallin yolk platelets (Ohlendorf, Wrenn and Banaszak, 1977) (fig. 1D). Finally, in stage VI, the accumulation of the materials required for embryonic development is completed and these oocytes may, in response to pituitary hormones undergo maturation and thus become eggs.

Are stage VI oocytes dormant cells?

White-banded oocytes (stage VI) are the type of oocytes most

frequently used in microinjection experiments. In this stage most morphological signs of activity have disappeared and for some time these oocytes were considered to be 'dormant'. For example a decrease of RNA synthesis was reported to occur in these oocytes (Brown and Littna, 1964; Brown, 1966) and an inhibitor of rRNA synthesis was reported to be present at this stage (Crippa et. al., 1972). However, more accurate measurements revealed, that stage VI oocytes are making as much RNA as stage IV oocytes. In both stages the main products are 18 and 28 S rRNA (LaMarca, Smith and Strobel, 1973; Dinauer and LaMarca, 1976). In fact, there appears to be a considerable turnover, about 1-2% per day, of the ribosomal RNA in stage VI oocytes (Leonard and LaMarca, 1975). Since oocytes at this stage contain about as much ribosomes as are present in the 30,000 cells of the swimming tadpole (Brown and Gurdon, 1964), a 1% turnover implies the synthesis of as many ribosomes as are present in 300 somatic cells. Coordinately with the ribosomal RNA, ribosomal proteins are synthesized and, since this represents only about 10% of the total proteins synthesized (Hallberg and Smith, 1975), it appears that stage VI oocytes are equally active in the synthesis of other cell components. In the interpretation of experiments with stage VI oocytes one should, therefore, consider, that although compared to their size and their ribosome content these giant cells are not very active, they are certainly not dormant cells.

Microinjection of oocytes

Like many other cell types, oocytes will tolerate the injection of fluid up to about 10% of their cell volume, i.e. up to 100 nl. Micro-

injection may be performed with the apparatus shown in fig. 2. Quantitative amounts of fluid can be delivered from the syringe, which is directly connected to a glass micropipette. This direct connection has the advantage over a set up in which syringe and micropipette are connected with flexible tubing, in that no delay in the delivery of the quantity of fluid due to the expansion and contraction of the tube wall, is observed. Syringe and micropipette should, however, be free of air bubbles, otherwise this advantage is lost. The micropipettes are made from 9 µl Drummond microcaps (calibrated glass capillaries) with a microelectrode puller (model PN-3W, Narishige Scientific Inst. Labs., Tokyo, Japan). They have a tip diameter of 10-20 µm. Since the injection needle will penetrate the oocyte about 0.5mm, the diameter immediately after the tip should not be much wider (fig. 2D). It is not necessary to sharpen the tip further on a microforge (Gurdon, 1974). The sample may be sucked in through the needle tip, provided it is not too viscous and that it does not contain dust particles, which can block the narrow passage in the needle. The fluid in the syringe (water) and the sample are separated with a drop of petroleumether or another water repellent fluid to prevent sample dilution. The micropipette can contain sufficient sample for 50-100 injections. Messenger RNAs and whole polyribosomes may be suspended either in distilled water or in Gurdon's injection solution (15 mM Tris/HCl pH 7.6 containing 88 mM NaCl and 15 mM KCl, ref. Gurdon, Lane, Woodland and Marbaix, 1971.) DNA is better dissolved in the latter injection solution.

Incubation and labeling of oocytes

A variety of media, ranging from simple salt solutions to complex nutritient media, have been used in culturing amphibian oocytes

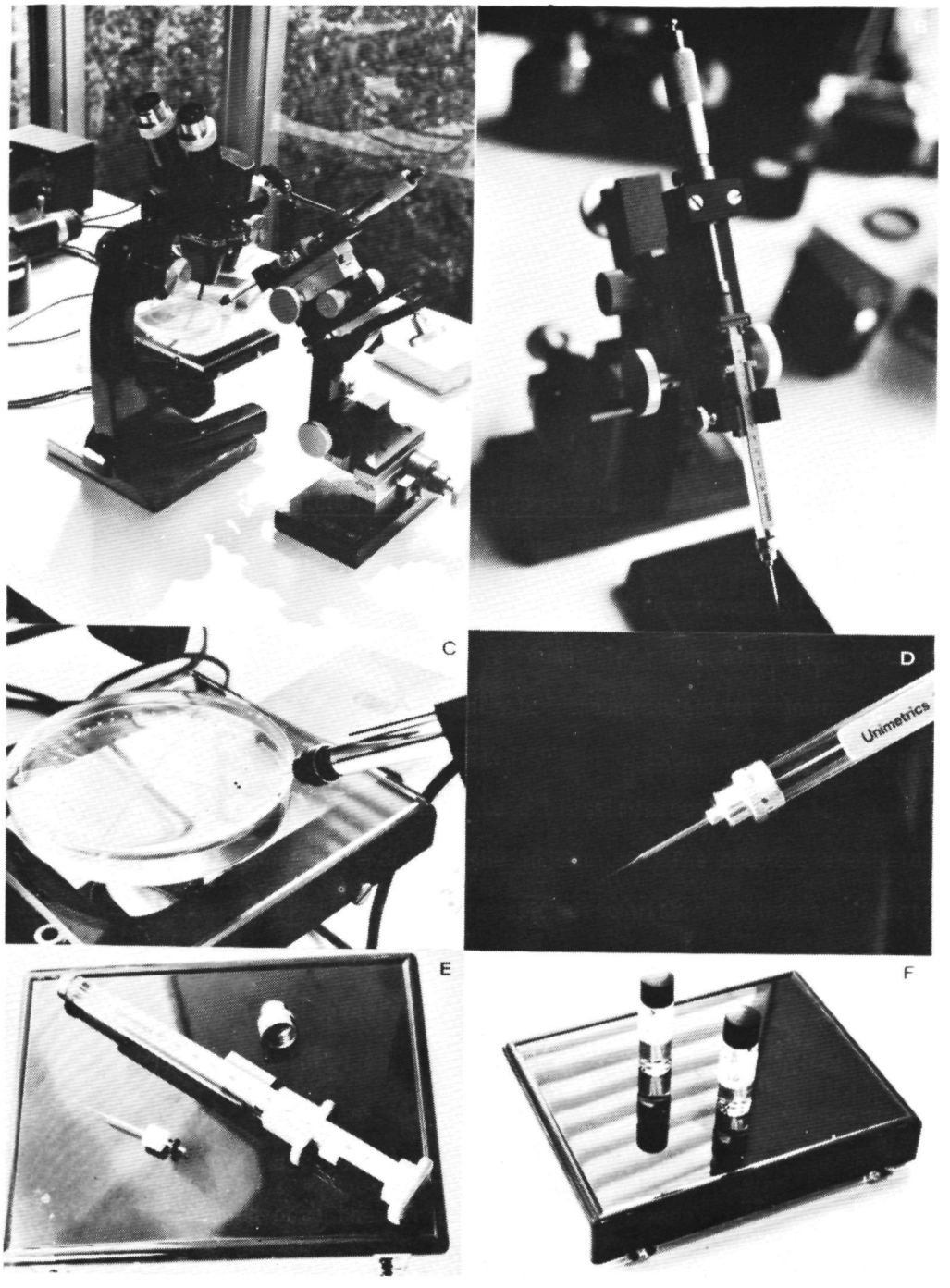

A
B
C
D
Unimetrics
E
F

Fig. 2. Oocyte microinjection apparatus:

(a) Overview. On the left the binocular microscope (Leitz) with a petridish containing the oocytes. On the right side the micromanipulator (Brinkmann MP IV), which holds the injection syringe. (b) The microinjection syringe (Unimetrix, 10 µl) is driven by a micrometer head (Agla MSO 6) and is attached to a glass micropipette. (c) Close-up of the injection needle during the injection. The oocytes are held in place in the petridish by a perspex disc with tiny holes (diameter 1.2 mm) in it. Oocytes are injected in the culture fluid. (d) Close-up of the injection micropipette, which consists of a glass capillary which narrows down to 10-20 µm at the tip. (e) The microinjection syringe taken apart. The glass micropipette is locked onto the syringe through a plastic cylinder to which an o-ring fits at each side. When the metal cap is screwed onto the syringe, the compressed o-rings effectively prevent all leakage. (f) Oocyte culture vessels.

(Barth and Barth, 1959; Auclair, 1961; Wolf and Quimby, 1964; Balls and Ruben, 1966; Merriam, 1966; Dettlaff, 1966; Gurdon, 1968; Jared and Wallace, 1969; Thomas, 1970; Gurdon and Laskey, 1970 a and b; Wallace, Jared, Dumont and Sega, 1973; Nakahashi and Yamana, 1974; Epping and Dumont 1976; Eppig and Steckman, 1976; Hallberg and Smith, 1976; Hanocq-Quertier, Baltus and Brachet, 1976; Colman, 1975; Bravo and Allende, 1976; Ford and Gurdon, 1977; Laskey, Mills, Gurdon and Partington, 1977; Cavalieri, Havell, Vilchek and Pestka, 1977; Wallace and Misulovin, 1978). In general, it should be kept in mind, that the optimal pH for oocytes is rather alkaline, pH = 7.6 - 8.5 (Bravo and Allende, 1976), and that the osmolarity of media for the culture of amphibian cells should be about 30% lower than for mammalian cells (Balls and Ruben, 1966). In our studies, Barth's medium (Barth and Barth, 1959), modified according to Gurdon (Gurdon, 1968), has always been used (table 2), but an apparently improved version of this medium, now including also phosphate salts and using HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) as a buffer, was recently introduced (Laskey et. al., 1977). Nutritient media present little advantage over buffered salt solutions, and then only during long term culture of defolliculated oocytes (Eppig and Dumont, 1976; Wallace and Misulovin, 1978), since oocytes are already supplied with amino acids from the yolk (Bravo, Salazar and Allende, 1976). In nutritient media bacterial infection is often a problem. Oocytes are easily cultured in small glass vessels (fig. 2F). The optimal temperature both for endogenous protein synthesis (Bravo and Allende, 1976) and for translation of exogenous (interferon) mRNA was found to be about 20$^{o}$C (Cavalieri et. al., 1977). In protein labeling studies, the radioactive amino acid is best

Table 2: MODIFIED BARTH'S MEDIUM

(Gurdon, J.B., 1968, J. Embryol. Exp. Morph. 20, 401)

Solution A:

| | | | |
|---|---|---|---|
| NaCl | 880 | mM | 51.42 g/l |
| KCl | 10 | mM | 745.6 mg/l |
| $Ca(NO_3)_2.4H_2O$ | 3.3 | mM | 779.3 mg/l |
| $CaCl_2.2H_2O$ | 4.1 | mM | 619.3 mg/l |
| $MgSO_4.7H_2O$ | 8.2 | mM | 2.02 g/l |

Solution B:

| | | | |
|---|---|---|---|
| $NaHCO_3$ | 24 | mM | 2.02 g/l |
| Tris | 20 | mM | 1.2 g/l |
| HCL | add until 1 part A plus 8 parts distilled water plus 1 part B have a pH of 7.6 | | |

Stock solutions A and B are sterilized by filtration through a 0.2 μm cellulose nitrate filter (Schleicher and Schuell, type BA 83) and stored at $4^{o}C$. The medium is made up by mixing:

1 part solution A, 8 parts distilled water and

1 part solution B (in this order).

Penicillin and streptomycin are added at final concentrations of 10 mg/l.

dissolved in the medium at 0.25-1.0 mCi/ml, since microinjected amino acids are quickly lost into the medium (Gurdon, et. al., 1971). In RNA labeling studies uridine or guanosine may be dissolved into the medium. Labeling with guanosine (stage VI oocytes do not synthesize DNA) is generally better than with uridine (Colman, 1974). Nucleoside triphosphates have to be injected into the cell, but fortunately they do not leak out (Dinauer and LaMarca, 1976., Anderson and Smith, 1977).

Microinjection of Xenopus eggs

In practical terms the main difference between oocytes and eggs, is the jelly layer which surrounds the egg. If it is removed (Froehlich, Bowder and Schultz, 1977), eggs may be injected and labeled just like oocytes. Alternatively the jelly layer may be made penetrable for the injection needle by UV irradiation (Gurdon, 1960; Gurdon et. al. 1971). In the latter case radioactive precursors must be injected into the eggs, since the uptake of substances from the medium is very poor. It is fortunate that leakage from such eggs is also much lower than from oocytes (Gurdon, 1971). Eggs have two advantages over oocytes. First, if the eggs are fertilized, the fate of the injected material may be followed during development of the embryo. Second, in contrast to oocytes, DNA microinjected into eggs is very efficiently replicated (Gurdon, Birnstiel and Speight, 1969). Presumably oocytes fail to do so, because they do not contain the necessary deoxynucleosides (Woodland and Pestell, 1972). Unfortunately, unfertilized eggs retain their metabolic activity only for a few hours (Gurdon, et. al., 1971; Mertz and Gurdon, 1977).

Analysis of the proteins synthesized in oocytes

Analysis of the proteins made by oocytes (or eggs) is complicated by the presence of the enormous amount of yolk protein and by the fat content of the oocyte. The fat may simply be removed by centrifugation or by ether extraction. The yolk platelets can be centrifuged down from the homogenate. The presence of some salt in the homogenization buffer reduces the loss of specific proteins in the yolk pellet (DeRobertis, Partington, Longthorne and Gurdon, 1977), however, in our experience a considerable (non-specific) loss of labeled protein still occurs. The supernatant proteins may then be analyzed, for example, by high resolution two-dimensional gel electrophoresis (DeRobertis et. al., 1977). When an immunoprecipitation method is to be used, the yolk protein may be dissolved by homogenizing the oocytes in a detergent containing buffer (Asselbergs, van Venrooij and Bloemendal, 1978), affording considerably better yield of radioactive protein.

Microinjection into the oocyte nucleus

A new and interesting development in molecular biology is, that purified genes are transcribed when microinjected in eggs or oocytes (Colman, 1975; Mertz and Gurdon, 1977; Kressmann, Clarkson, Telford and Birnstiel, 1977, 1978; Kressmann, Clarkson, Protta and Birnstiel, 1978; DeRobertis and Mertz, 1977; Brown and Gurdon, 1978; Gurdon, 1978; Rungger and Tuerler, 1978; Trendelenburg and Gurdon, 1978; DeRobertis and Olson, 1979). It has even been shown, that some of the transcribed sequences are processed to mature mRNAs which subsequently is translated (DeRobertis and Mertz, 1977; Kressmann et. al., 1977; Rungger and Tuerler, 1978). In these experiments it was found to be essential to inject the DNA into the nucleus. DNA injected into the cytoplasm is

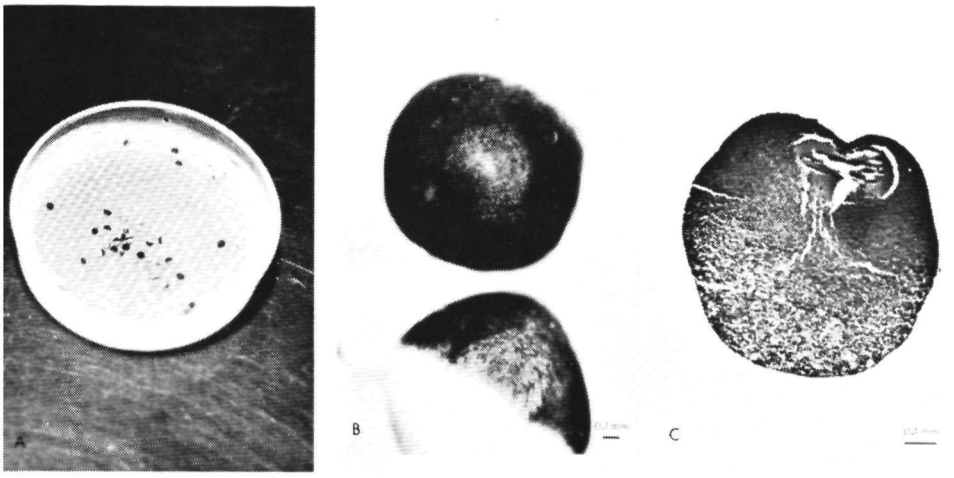



Fig. 3: Microinjection in the oocyte nucleus

(A) Oocytes in petridish on a plastic gauze used to keep them in position during centrifugation. (B) Oocytes immediately after centrifugation. The lighter area in the black hemisphere of the oocyte indicates the position of the nucleus. (C) Cross-section of centrifugated oocyte. The nucleus has been pushed upwards against the pigment layer of the animal hemisphere, while the yolk platelets, because of their higher density, have been centrifuged down to the vegetal pole. The disruption of the nucleus is probably a fixation artifact.

not transcribed (Mertz and Gurdon, 1977; Kressmann et. al., 1978a), but degraded (Wyllie, Gurdon and Price, 1977; Wyllie, Laskey, Finch and Gurdon, 1978). The DNA injected into the nucleus is converted into chromatin (Wyllie et. al., 1978), in which form it is apparently protected from degradation. In this respect it should be mentioned that oocytes are unique in that they contain a large pool of free histones (Woodland and Adamson, 1977). Transcription of the following microinjected DNAs has been demonstrated so far: Simian Virus 40 DNA, Adenovirus DNA, cloned Drosophila histone DNA (Mertz and Gurdon, 1977), cloned sea urchin histon DNA and cloned Xenopus $tDNA_i^{Met}$ (Kressmann et. al., 1977), cloned Xenopus 5 S rDNA (Brown and Gurdon, 1978) and Xenopus (18 + 28 S) rDNA (Wyllie et. al., 1978; Trendelenburg and Gurdon, 1978), all eukaryotic, plus the following genes of prokaryotic origin: Ø80plac, ØX174RF and ColE1 plasmid DNA (Mertz and Gurdon, 1977). Transcription by RNA polymerases I (18 and 28 S rRNA) and III (5 S rRNA and tRNA) was shown to occur on the correct DNA strand and to yield the correct products. The data on the fidelity of transcription by RNA polymerase II (messenger RNA) and subsequent processing of the transcripts are at the moment inconclusive. Some of the products were apparently processed correctly, since the correct translation products were detected after injection of Simian Virus 40 DNA (Mertz and Gurdon, 1977, Rungger and Tuerler, 1978) Drosophila histon DNA (Mertz and Gurdon, 1977) and Adenovirus DNA (Asselbergs et. al., unpublished). However, it was also reported that most of the transcription products of sea urchin histon DNA were either larger or smaller than mature histone mRNA (Kressmann et. al., 1978). In addition a large amount of RNA seemed to be transcribed from the non-coding DNA strand. In all cases the yield of

translation products was much lower than expected, if all transcripts were also translated.

Technically, the major problem encountered in nucleus injection experiments is that of locating the nucleus in the opaque oocyte. One approach was made by Gurdon (1976), who demonstrated that the relatively large germinal vesicle (diameter about 0.1 mm., fig. 1B) can be found by piercing the animal pole of the oocyte, while aiming the needle at the center of the oocyte and squeezing both sides of the oocyte with a pair of forceps. An alternative, much easier procedure, was developed by Kressmann et. al., (1977). When Xenopus oocytes are centrifuged gently (10 min, 600-1000 x g, 18$^{o}$C) the oocyte nucleus floats towards the surface of the cell. In doing so, they displace pigment granules causing a lighter spot to appear on the oocyte surface (fig. 3 B) which frequently is surrounded by a darker ring. The ring remains visible for about an hour. Before centrifugation oocytes are placed on a plastic grid (mesh size 0.8 mm) which is fixed (with chloroform) to the bottom of a plastic petri dish (fig. 3A). The oocytes are turned manually so that the animal pole (the dark hemisphere) is facing upwards. For each batch of oocytes the optimal centrifugation speed is different and in general lower speeds are necessary, if the oocytes have been cultured in vitro for longer periods. During centrifugation the oocytes become fixed in the grid (fig. 3B). The oocytes may be injected while in the grid or they may be taken out before injection. The needle is now aimed at the center of the light spot, while care is taken not to insert the needle too deeply into the oocyte. About 20 nl can be injected. Further culture and labeling of the oocytes is performed as described above.

References

1. Anderson, D.M. and Smith, L.D. (1977), Cell 11, 663-671.

2. Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H. (1978), Eur. J. Biochem. 87, 517-524.

3. Auclair, W. (1961), Nature 192, 467-468.

4. Avery, O.T., MacLeod, C.M. and McCarthy, M. (1944), J. Exptl. Med. 79, 137-158.

5. Balls, M. and Ruben, L.N. (1966), Exptl. Cell Res. 43, 694-695.

6. Barth, L.G. and Barth, L.J. (1959), J. Embryol. Exp. Morphol. 16, 183-195.

7. Bravo, R., and Allende, J.E. (1976, Arch. Biochem. Biophys., 172, 648-653.

8. Bravo, R., Salazar, F. and Allende, J.E. (1976), Exptl. Cell Res. 103, 169-174.

9. Brown, D.D. (1966), Natl. Cancer Inst. Monographs, 23, 297-225.

10. Brown, D.D. and Gurdon, J.B. (1978), Proc. Natl. Acad. Sci. USA, 75, 2849-2853.

11. Brown D.D. and Littna, E. (1964), J. Mol. Biol. 8, 688-695.

12. Cabada, M.D., Darnbrough, C., Ford, P.J. and Turner, P.C. (1977) Dev. Biol. 57, 427-439.

13. Cairns, E., Gschwender, H.H., Primke, M., Yamakawa, M., Traub, P., and Schweiger, H.G. (1978), Proc. Natl. Acad. Sci. USA 75, 5557-5559.

14. Cavalieri, R.L., Havell, E.A., Vilchek, J. and Pestka, S. (1977) Proc. Natl. Acad. Sci. USA 74, 3287-3291.

15. Coggins, L.W. (1973) J. Cell Sci. 12, 71-

16. Colman, A. (1975), Eur. J. Biochem. 57, 85-96.

17. Crippa, M. et. al., (19 ) in: "Oogenesis" (Biggers J.D. and Schuetz, A.W., eds.), pp. 193-214, Univ. Press, Baltimore, Maryland USA.

18. Davidson, E.H. (1976) Gene activity in early development, 2nd edition, Acad. Press, New York.

19. Denis, H. (1977) Biologie cellulaire 28, 87-92.

20. DeRobertis, E.M. and Olson, M.V. (1979) Nature 278, 137-143.

21. DeRobertis, E.M., Partington, G.A., Longthorne, R.F. and Gurdon, J.B. (1977), J. Embryol. Exp. Morph. 40, 199-214.

22. Detlaff, T.A. (1966), J. Embryol. Exp. Morph. 16, 183-195.

23. Dimitriados, G. (1978) Nature 274, 923-924.

24. Dinauer, M.C. and LaMarca, M.J. (1976), Exptl. Cell Res. 101, 122-126.

25. Dumont, J.N. (1972), J. Morphol., 136, 153-180.

26. Dumont, J.N. and Brummet, A.R. (1978) J. Morphol. 155, 73-98.

27. Eppig, J.J. and Dumont, J.N. (1976) In Vitro 12, 418-425.

28. Eppig, J.J. and Steckman, M.L. (1976) In Vitro 12, 173-179.

29. Ford, C.C. and Gurdon, J.B. (1977) J. Embryol. Exp. Morphol. 37, 203-209.

30. Froehlich, J.P., Browder, L.W. and Schultz, G.A. (1977) Dev. Biol. 56, 356-371.

31. Graessmann, A. and Graessmann, M. (1971) Hoppe Zeyler Z Physiol. Chem. 25, 527-532.

31a. Graessmann, M. and Graessmann, A. (1976) Proc. Natl. Acad. Sci. USA 73, 366-370.

32. Gurdon, J.B. (1960) Quart. J. Microscopical Sci. 101, 299-311.

33. Gurdon, J.B. (1968) J. Embryol. Exp. Morphol. 20, 401-

34. Gurdon, J.B. (1974) The control of gene expression in animal development, Oxford and Harvard University Presses.

35. Gurdon, J.B. (1976) J. Embryol. Exp. Morphol. 36, 523-540.

36. Gurdon, J.B. (1978) Proc. Roy Soc. London 198, 211-247.

37. Gurdon, J.B., Birnstiel, M.L. and Speight, V.A. (1969) Biochim. Biophys. Acta. 174, 614-628.

38. Gurdon, J.B., Lane, C.D., Woodland, H.R. and Marbaix, G. (1971) Nature 23, 177-182.

39. Gurdon, J.B. and Laskey, R.A. (1970a) J. Embryol. Exp. Morphol. 24, 227-248.

40. Gurdon, J.B. and Laskey, R.A. (1970b) J. Embryol. Exp. Morphol. 24, 249-255.

41. Hallberg, R.L. and Smith, D.C. (1975) Dev. Biol. 42, 40-52.

42. Hallberg, R.L. and Smith, D.C. (1976) Dev. Biol. 48, 308-316.

43. Hanocq-Quertier, J., Balthus, E. and Brachet, J. (1976) Proc. Natl. Acad. Sci. USA 73, 2028-2032.

44. Jared, D.W. and Wallace, R.A. (1969) Exptl. Cell Res. 57, 454-457.

45. Knowles, J.K.C., Lipps, H.J. and Nook, A. (1978) Biochem. Biophys. Res. Commun. 80, 897-904.

46. Kressmann, A., Clarkson, S.G., Telford, J.L. and Birnstiel, M.J. (1977) Cold Spring Harbor Symp. Quant. Biol. 47, 1077-1082.

47. Kressmann, A., Clarkson, S.G., Telford, T.L. and Birnstiel, M.D. (1978) Nucleic Acids Res.

48. Kressmann, A., Clarkson, S.G., Pirotta, V. and Birnstiel, M.L. (1978) Proc. Natl. Acad. Sci. USA 75, 1176-1180.

49. Kriegler, M.P. and Livingston, D.M. (1977) Somatic Cell Genetics 3, 603-611.

50. LaMarca, M.J., Smith, L.D., and Strobel, M.J. (1973) Dev. Biol. 34, 106-108.

51. Laskey, R.A., Mills, A.D., Gurdon, J.B. and Partington, G.A. (1977) Cell 11, 345-352.

52. Leonard D.A. and LaMarca, M.J. (1975) Dev. Biol. 45, 199-202.

53. Loyter, A., Zakai, N. and Kulka, R.G. (1975) J. Cell Biol. 66, 292-304.

54. Merriam, R.W. (1966) Exptl. Cell Res. 42, 340-347.

55. Mertz, J.E. and Gurdon, J.E. (1977) Proc. Natl. Acad. Sci. USA 74, 1502-1506.

56. Nakahaski, T. and Yamana, K. (1974) Expt. Cell Res. 88, 241-245.

57. Ohlendorf, D.H. (1977) J. Biol. Chem. 252, 7992-8001.

58. Ohlendorf, D.H., Wren, R.F. and Banaszak, L.J. (1977) Nature 272, 28-32.

59. Ostro, M.J., Giacomo, D., Lavelle, D., Paxton, W., and Dray, S. (1978) Nature 274, 921-923.

60. Picard, B. and Wegnez, M. (1979) Proc. Natl. Acad. Sci. USA 76, 241-245.

61. Rosbash, M. and Ford, P.J. (1974) J. Mol. Biol. 85, 87-101.

62. Rungger, D. and Tuerler, H. (1978) Proc. Natl. Acad. Sci. USA 75, 6073-6077.

63. Stacey, D.W. and Allfrey, V.G. (1976) Cell 9, 725-732.

64. Stacey, D.W., Allfrey, V.G. and Hanafusa, H. (1977) Proc. Natl. Acad. Sci. USA 74, 1614-1618.

65. Tata, J.R. (1976) Cell 9, 1-14.

66. Thomas, C. (1970) Biochim. Biophys. Acta 224, 99-113.

67. Trendelenburg, M.F., and Gurdon, J.B. (1978) Nature 276, 292-294.

68. Yang, S.F. and Niu, M.C. (1977) Proc. Natl. Acad. Sci. USA 74, 1894-1898.

69. Tuohimaa, P. and Soederling, E. (1976) Acta Endocrinol. 81, 593-597.

70. Wallace, R.A. and Jared, D.W. (1976) J. Cell Biol. 69, 345-351.

71. Wallace, R.A., Jared, D.W., Dumont, J.N. and Sega, M.W. (1973) J. Exp. Zool. 184, 321-334.

72. Wallace, R.A. and Misulovin, Z. (1978) Proc. Natl. Acad. Sci. USA 75, 5534-5538.

73. Webb, A.C. and Smith, L.D. (1977) Dev. Biol. 56, 219-225.

74. Wigler, M., Pellicer, A., Silverstein, S. and Axel, R. (1978) Cell 14, 724-731.

75. Wolf, K. and Quimby, M.C. (1964) Science 144, 1578-1580.

76. Woodland, H.R. (1974) Dev. Biol. 40, 90-101.

77. Woodland, H.R. and Adamson, E.D. (1977) Dev. Biol. 57, 118-135.

78. Woodland, H.R. and Pestell, R.Q.W. (1972) Biochem. J. 127, 595-605.

79. Wyllie, A.H., Gurdon, J.B. and Price, J. (1977) Nature 268, 150-152.

80. Wyllie, A.H., Laskey, R.A., Finch, J. and Gurdon (1978) Dev. Biol. 64, 178-188.

# CHAPTER III

## MICRO-INJECTION OF MESSENGER RNA INTO AMPHIBIAN OOCYTES:

## REGULATION OF MESSENGER RNA TRANSLATION

III - 1

# MICRO-INJECTION OF MESSENGER RNA INTO AMPHIBIAN OOCYTES AS PROBE FOR THE POST-TRANSCRIPTIONAL CONTROL OF PROTEIN SYNTHESIS

## III-1 MICRO-INJECTION OF MESSENGER RNA INTO LIVING AMPHIBIAN OOCYTES AS PROBE FOR THE POST-TRANSCRIPTIONAL CONTROL OF PROTEIN SYNTHESIS

Eukaryotic mRNAs generally have longer half-lives as their prokaryotic counterparts. Hence, changes of the rate of mRNA synthesis have only effect on protein synthesis after prolonged periods of time. The cell type which is the main subject of the present review, the amphibian oocyte, is a particularly good example of this phenomenon. If transcription is blocked in these cells (for example by enucleation or with antibiotics), neither the rate of protein synthesis nor the specificity seem to be affected. In these cells protein synthesis is apparently subject to some form of cytoplasmic control. Post-transcriptional and translational control of protein synthesis, primarily as revealed from work with cell-free extracts, has been the subject of two recent reviews (Lodish, 1976, Revel and Groner, 1978). In this article emphasis is given to those features of post-transcriptional control of protein synthesis which have been discovered by means of micro-injection of mRNA into living cells.

### Specificity of translation inside living cells

Micro-injection into amphibian eggs and oocytes has been used as an assay method for a wide variety of mRNAs (previous reviews: Gurdon, 1974; Lane and Knowland, 1975). Fidelity of translation of heterologous mRNAs has been demonstrated repeatedly with peptide maps (May and Glenn, 1974; Mach, Faust, Vassali and Rungger, 1973; Marbaix and Lane, 1972; Lane and Knowland, 1975) and partial amino acid sequences (Kindas-Mugge, Lane and Kreil, 1974) of the translation products. A list of the mRNAs translated in amphibian eggs and oocytes, as complete as the knowledge of the author allows, is given at the end of this article (tables 3

and 4). It appears, that the translational machinery of these cells is compatible with cytoplasmic mRNAs (table 3) and virus specific RNAs (table 4) from many tissues of either plant or animal origin. Absolute specificity for certain types of eukaryotic mRNA is also not generated during embryonic development since globin mRNA, micro-injected into fertilized Xenopus eggs, continues to be translated in the descendants, even when they have differentiated into muscle or nerve cells (Woodland, Gurdon and Lingrel, 1974). In a few cases heterologous messenger RNA has been transferred directly into adult cells. For example, melanin synthesis has been reported to occur in muscle cells injected with the RNA from Harding Passey melanoma cells (Graessmann and Graessmann, 1971) and chicken globin synthesis has been demonstrated in HeLa cells micro-injected with purified mRNA (Stacey and Allfrey, 1976). Heterologous mRNA such as coding for chicken avidin (Tuohimaa and Soederling, 1976) or for serum albumin of various species (Yang and Niu, 1977), were absorbed from the lumen of the mouse uterus and subsequently become translated by the cells of the uterine wall. Finally, rabbit globin mRNA can be encapsulated in liposomes and in this form has been introduced and translated in cultured human lymphocytes (Dimitriados, 1978) and human epithelial carcinoma cells (Ostro, Giacomo, Lavelle, Paxton and Dray, 1978). Animal virus RNA has even been translated in living plant cells (Cairns, Gschwender, Primke, Yamakawa, Traub and Schweiger, 1978). The discrimination between cytoplasmic mRNA species from different cell types, which has been observed in some cell-free systems (Lodish, 1976; Revel and Groner, 1978), is apparently not paralleled in vivo.

Prokaryotic messenger RNAs may be translated correctly by eukaryotic (80 S) ribosomes in cell-free systems, albeit with very low

efficiency (Herrlich and Schweiger, 1978). In Xenopus oocytes the efficiency of translation of prokaryotic f2 bacteriophage RNA must also be extremely low, since no translation product at all could be detected (Gurdon, Lane, Woodland and Marbaix, 1971). Translation of mitochondrial mRNA, which resembles prokaryotic mRNA in many aspects, has been claimed (Eggit and Scragg, 1975) and denied (Moorman, Verkley, Asselbergs and Grivell, 1977). In both cases immunoprecipitation was used to detect the synthesis of polypeptides encoded by the injected mitochondrial RNA. In the experiments of Eggit and Scragg (1975) the amount of immunoprecipitated polypeptides was only increased twofold over the background and the precipitated polypeptides were insufficiently characterized. Moorman et al (1977) reported the translation in an E.coli cell-free system of purified 11 S mitochondrial mRNA into products immunoprecipitable with antiserum against subunits 1 and 2 of cytochrome c oxidase. However, no translation of this mRNA was observed in eukaryotic cell-free systems and in oocytes. The evidence, that translation of mitochondrial RNA by extra-mitochondrial ribosomes is possible under in vivo conditions, is therefore at present debatable.

Synthetic polyribonucleotides are not translated in oocytes. Homopolymers and most types of heteropolymers also do not affect translation of other (endogenous or exogenous) mRNAs in oocytes. $AUG(U)_n$ strongly inhibits all protein synthesis. On the other hand, poly (G,U), poly (A,G,U) and poly (A,C,G,U) only inhibit translation of microinjected (globin) mRNA, when simultaneously injected, but do not inhibit endogenous protein synthesis (Woodland and Ayers, 1974).

Most eukaryotic mRNAs are synthesized as large nuclear precursor RNAs (hnRNA), which are subsequently shortened by the deletion of

internal pieces of the polynucleotide chain, a process called splicing (review: Crick, 1979). Since frequently, for example in the case of globin mRNA (Jeffreys and Flavell, 1977; Kinniburgh, Mertz and Ross, 1978), an intervening sequence within the coding part is removed from the pre-mRNA, it is theoretically impossible to translate this pre-mRNA into a normal protein prior to its processing. Nevertheless, several reports claim the detection of correct translation products after micro-injection of purified hnRNA into oocytes (Rollins, and Flickinger, 1972; Stevens and Williamson, 1972, 1973 a and b; Williamson, Drewienkiewcz and Paul, 1973). In view of the very low content of pre-mRNA in the cell nucleus (less than 1% of all globin mRNA-sequences are in pre-mRNA (Ross and Knecht, 1978)), this is most likely explained by contamination of the HnRNA with mature mRNA (Lane, Gregory, Iazumi and Scherrer, 1973). However, the possibility that oocytes can process micro-injected pre-mRNA and subsequently translate it, cannot be excluded, since oocytes have been shown to "splice" newly made SV40 pre-mRNA after intranuclear injection of SV40 DNA (Rungger and Tuerler, 1978).

Efficiency of translation of micro-injected messenger RNA

Two procedures may be used to obtain a rough estimate of the number of polypeptides synthesized per injected messenger RNA molecule. In method A, which is applicable to all translation products of known molecular weight, all polypeptides synthesized by the oocyte are uniformly labeled using a mixture of radioactive amino acids (Laskey, Mills, Gurdon and Partington, 1977). From the % foreign translation product and the known synthetic activity of the stage VI oocyte (table 2), one can calculate the amount of polypeptide synthesized. If not only the size, but also the amino acid composition of the translation

Table 1: EFFICIENCY OF TRANSLATION OF MESSENGER RNA INJECTED IN XENOPUS OOCYTES

| nr. | encoded polypeptide | pmol mRNA injected | pmol protein synthesized $h^{-1}$ | polypeptides/ mRNA, h | estimation method | reference |
|---|---|---|---|---|---|---|
| 1. | rabbit β-globin | 0.0072[1]) | 0.18 | 25 | B | Gurdon, (1973) |
| 2. | rabbit β-globin | 0.0162 | 0.288[2]) | 17.5 | B | Asselbergs et al (1978d) |
| 3. | rabbit β-globin | 0.0102[3]) | 0.097[2])3) | 9.6 | B | Asselbergs et al.(1978d) |
| 4. | rabbit β-globin | 0.0102 | 0.087 (5.9%)[4]) | 8.6 | A | Asselbergs, unpublished |
| 5. | duck α β-globin | 0.080 | 0.160 | 2.0 | B | Lane et al. (1973) |
| 6. | calf $\alpha A_2$-crystallin | 0.0022[5]) | 0.047 | 21.5 | B | Asselbergs et al. (1978d) |
| 7. | calf $\alpha A_2$-crystallin | 0.0011[3]) | 0.022 | 20.5 | B | Asselbergs et al. (1978d) |
| 8. | calf $\alpha A_2$-crystallin | 0.001[5]) | 0.028 (2.5%)[4]) | 25.8 | A | Asselbergs, unpublished |
| 9. | chicken ovalbumin | 0.0118 | 0.129 | 10.9 | B | Chan et al., (1976) |
| 10. | Rauscher Murine leukemia viral RNA | 0.0036[6]) | 0.0068 (2.0%)[4]) | 1.9 | A | Asselbergs et al., (1979c) |

1) data have been recalculated, assuming 41% of the polysomal globin mRNA in rabbits is β-globin mRNA
2) data recalculated, taking into account, that 66% of the total globin synthesized by oocytes in absence of hemin was β-globin.
3) 9 S globin mRNA and 14 S $\alpha A_2$-crystallin mRNA were injected together in the same oocytes.
4) % of total oocyte protein synthesis (assayed with a mixture of amino acids)
5) values 6 and 8 were obtained in seperate batches of oocytes from the same frog.
6) R-MuLV 70 S RNA complex consists of two identical 3.5 x $10^6$ dalton translatable RNA species.

product is known, the translational efficiency is more accurately measured using method B, in which oocytes are labeled with a single amino acid. The amount of foreign translation product synthesized is calculated from the radioactivity incorporated into the encoded polypeptide and the intracellular specific activity of the radioactive amino acid. The latter parameter is calculated from the total amount of the amino acid present in the oocytes, measured with an amino acid analyzer, and the amount of free radioactive amino acid in the cell (Gurdon et al., 1971, Asselbergs, van Venrooij and Bloemendal, 1978f). In such calculations it is assumed that the specific activity of the kinetic pool is the same as that of the total amino acid pool. Since usually long labeling periods are used, this assumption seems generally valid, as has been demonstrated in the case of histidine (Lane, Gregory and Morel, 1973). The two estimation methods give comparable results in the case of $\alpha A_2$-crystallin mRNA (cf. table 1 nrs. 6 and 8). At low concentrations of the injected mRNA, synthesis of up to 25 polypeptides/mRNA has been observed (table 1). This means that, for example, rabbit β-globin mRNA is translated with an efficiency only fourfold lower than it is in reticulocytes cultured at 20°C (Hunt, Hunter and Munro, 1969) (the usual incubation temperature for Xenopus oocytes is 19°C). Translation of chicken ovalbumin mRNA in oocytes must be as efficient as it is in the oviduct, taking into account the effect of the lower incubation temperature (Chan, Kohler and O'Malley, 1976). It should be pointed out, that the values of table 1 are lowest estimates, since they are based on the assumption that all injected mRNA molecules are translated. Furthermore, at higher mRNA concentration the efficiency of translation decreases due to competition between the injected templates (section

III-3: Asselbergs, van Venrooij and Bloemendal (1979d); Laskey, Mills, Gurdon and Partington (1977)).

The efficiency of translation of heterologous mRNA in oocytes is probably equal or better than the initial efficiency in most cell-free systems (Gurdon, Lane, Woodland and Marbaix, 1971). Moreover, translation proceeds for a much longer period of time, which can result in the accumulation in 24 h (in the case of interferon mRNA) of 500 times as much of the translation product than synthesized during a typical incubation in a cell-free system (Reynolds, Premkumar and Pitha, 1975). It is this high yield of translation products which allows detection by their enzymatic or other biological activities, for example in the case of the flower style incompatibility protein, interferon and β-glucuronidase (table 3).

What factors limit protein synthesis in oocytes?

In amphibian oocytes the remarkable situation exists, that there are 50-100 times more ribosomes present in the cell than are actually engaged in protein synthesis (table 2). These ribosomes are probably capable to carry out protein synthesis since they were active in a poly(U) programmed cell-free system (Cox, Ford and Pratt, 1970; van der Saag, Vlak and de Greef, 1976). Furthermore, translation of microinjected rabbit globin mRNA in cells of the 8-16 cell stage of development of Pleurodeles walthii (newt) eggs is not a function of the total concentration of ribosomes, since the amount of globin synthesized in mRNA injected micromeres is equal to that synthesized in macromeres, despite the fact that micromeres contain much more ribosomes (Brachet, Huez and Hubert, 1973).

TABLE 2: PROTEIN SYNTHETIC APPARATUS OF A FULL-GROWN XENOPUS OOCYTE

| | | |
|---|---|---|
| total RNA content | 4000 ng | (Davidson, 1976) |
| ribosomal RNA content | 3600 ng | |
| ribosome content | 1.67 pmol | |
| ribosome content | ca. $10^{12}$ | |
| % ribosomes as polyribosomes (=engaged in protein synthesis) | 1-2% | (Woodland, 1974) |
| poly-A containing mRNA content | ca. 2 x $10^{11}$ | (Cabada et al., 1977) |
| Poly-A containing mRNA content | 0.3 pmol | |
| % mRNA in polyribosomes | < 10% | (Roshbash and Ford, 1974) |
| protein synthesis | 19-25 ng $h^{-1}$ | (Davidson, 1976) |

Similarly, oocytes contain much more mRNA than is actually used in protein synthesis (table 2). This quiescent mRNA in excess is not functionally impaired, when assayed in vitro (Darnborough and Ford, 1976). Further evidence that in oocytes the availability of mRNA does not restrict protein synthesis in oocytes, comes from the observation, that injection of extra messenger in oocytes does not increase total protein synthesis (Lane et al., 1973; Laskey et al., 1977; Asselbergs et al., 1979d) for the limited translational capacity of these cells (see note).

NOTE: *In some experiments, in which oocytes were labeled with ($^3H$)-histidine, microinjection of extra (rabbit globin) mRNA enhanced incorporation of radioactive amino acid (Moar, Gurdon, Lane and Marbaix,*

*1971). On the other hand, if ($^{35}S$)methionine was used to label oocytes, a decrease in incorporation was observed (Laskey et al., 1977). These effects have been attributed to the fact, that oocytes injected with globin mRNA switched from the synthesis of their own proteins to the synthesis of a histidine-rich but methionine-poor protein (globin). A more balanced assay for the proteins synthesized by oocytes was obtained, if a mixture of radioactive amino acids rather then a single labeled amino acid was used to label oocytes (Laskey et al., 1977). Thus, the enhanced incorporation of a single labeled amino acid observed after injection of other mRNA species, notably immunoglobulin light chain mRNA (Jilka, Cavalieri, Yaffe and Pestka, 1977), ovalbumin mRNA (Chan et al., 1976) and tyrosine amino transferase mRNA (Nickol, Lee, Hollinger and Kenny, 1976), may not reflect a true enhancement of protein synthesis. Enhanced incorporation of ($^{3}H$)histidine in oocytes injected with rabbit globin mRNA was not observed in most of the author's experiments (unpublished) and the stimulatory effect may therefore be dependent to some extent on the batch of oocytes used.*

---

Several lines of evidence indicate, that the initiation of protein synthesis (the ribosome binding process) is not limiting mRNA translation in oocytes. Messenger RNAs coding for rabbit α- and β-globin differ in their intrinsic rate of ribosome binding both in intact reticulocytes and in many cell-free systems (Lodish, 1971; 1976; McKeehan, 1974). At high mRNA concentrations, when the two messengers are competing for the limiting initiation capacity of the translation system, β-globin mRNA, having a higher ribosome binding activity, is translated in excess over α-globin mRNA (McKeehan, 1974). However, when assayed in Xenopus oocytes in the absence of coinjected hemin, the

ratio of the synthesized α- and β-globin remains unchanged, when the intracellular messenger RNA concentration is increased (Gurdon, Lingrel and Marbaix, 1973; Lane, Gurdon and Woodland, 1974, Lingrel and Woodland, 1974). In the presence of coinjected hemin (which enhances α-globin synthesis in oocytes (below)) the α/β-globin ratio decreased at very high (48 ng mRNA/cell) (Lane et al, 1974) but not at moderate mRNA concentrations (up to 20 ng/cell). (Giglioni, Gianni, Comi, Ottolenghi and Rungger, 1973).

Similarly, the messenger RNAs encoding the different calf lens crystallins are thought to have different intrinsic rates of ribosome binding, as judged from experiments in reticulocyte cell-free systems (Asselbergs, Peters, van Venrooij and Bloemendal, 1978b-e). Also in this case, the ratios of the different crystallins remain unchanged during messenger RNA competition experiments in Xenopus oocytes (Asselbergs et al., 1979d). The messenger RNAs in oocytes are, therefore, competing for a factor with properties different from the (initiation) factors, which restrict protein synthesis in many other cell types and in cell-free translation systems. A direct indication, that initiation factors are not limiting protein synthesis in oocytes, comes from experiments, in which initiation factors were injected into oocytes (Marbaix and Gurdon, 1972). A 0.5 M KCl wash of ribosomes from Xenopus oocytes or rabbit reticulocytes did not stimulate the synthesis of oocyte proteins or rabbit globin significantly.

Oocytes contain little transfer RNA as compared to their tremendous content of ribosomes, but microinjection of extra tRNA did not enhance mRNA translation in oocytes (Gurdon, 1973). An insufficient capacity of the oocyte machinery for amino acylation of the available tRNA is

also very unlikely, since efficient charging of tRNA still occurred, when much more tRNA was injected than the oocyte itself already contained (Gatica, Tarrago, Allende and Allende, 1977).

The restraint on protein synthesis in oocytes could be overcome, if whole reticulocyte polyribosomes rather than naked mRNA was injected (Laskey et al., 1977). After injection of polyribosomes total protein synthesis had increased and synthesis of endogenous proteins of the oocytes had not decreased, indicating, that the messenger RNA contained in the injected polyribosomes, did not compete with the endogenous mRNAs of the oocyte. Injected in the form of polyribosomes globin mRNA did also not compete with co-injected Encephalomyocarditis viral RNA (Laskey et al., 1977). Therefore, protein synthesis in oocytes is probably limited by some component normally present on polyribosomes.

Size of polyribosomes translating injected globin mRNA

The nature of the factors restricting translation of a certain mRNA species in a living cell may be revealed by monitoring the size of the polyribosomes synthesizing the particular polypeptides (Palmiter, 1974; Lodish and Froshauer, 1977). For example, if in rabbit reticulocytes the initiation of polypeptide synthesis is inhibited, the globin messengers are found associated with fewer ribosomes. On the other hand, if factors involved in elongation become more limiting, polyribosome size will increase (Lodish, 1971). Thus it was concluded that in intact reticulocytes the initiation step is limiting protein synthesis (Lodish, 1974), whereas in chicken oviduct cells elongation is limiting (Palmiter and Schimke, 1973). In reticulocytes β-globin chains are predominantly synthesized on tetra- and pentasomes, α-globin chains are made on tri- and tetrasomes (Lodish, 1971; Hunt et al., 1969). This difference

in the size of polyribosomes reflects the different intrinsic rates of initiation of the α- and β-globin mRNAs respectively (Lodish, 1971; 1974). In Xenopus oocytes rabbit α- and β-globin are synthesized on polyribosomes containing 3-7 ribosomes (Lingrel and Woodland, 1974). Apparently the ratio between initiation and elongation of the globin polypeptides is very similar to that observed in reticulocytes. Unfortunately it is not known, whether in oocytes α-globin mRNA is also translated on slightly smaller polyribosomes than β-globin mRNA. The size of the globin synthesizing polyribosomes did not change, when 8 times as much globin mRNA was injected into the oocytes (Lingrel and Woodland, 1974), as would have been the case, if a step in the initiation or in the elongation of the globin polypeptides exclusively had been limiting translation of the messenger RNA. Thus, in the presence of excess mRNA, the translational machinery is not shared equally between all mRNA and the rate of protein synthesis is regulated rather by the number of mRNAs that is being translated.

Compartmentation of the mRNA pool in oocytes

Most of the messenger RNA in oocytes of amphibia (and of many other organisms as well) is not present in polyribosomes (Davidson, 1976; table 2). The question has been raised, as to whether all this mRNA is available for translation. Part of it might be present in temporarily inactivated ("masked") mRNP particles (by some authors referred to as informosomes (Spirin, 1969)) to be activated at different moments during maturation of the oocyte and subsequent embryonic development (Davidson, 1976). For example, the tenfold increase in the rate of histone synthesis at the time of maturation (Adamson and Woodland, 1977) occurs also in total absence of mRNA synthesis and probably is caused by

activation of masked histone message.

In Xenopus more than 90% of the total oocyte mRNA is found in the cytosol (Rosbash and Ford, 1974), so in compartment B and C (fig. 1) and only about 10% in the polyribosomes (compartment D, fig. 1). Microinjected mRNA has to compete with the endogenous mRNAs for the limited translational capacity of the cell (Laskey et al., 1977). In part 3 of this chapter it is demonstrated, that injected message and endogenous message are selected with equal probability by the translational apparatus. It could, therefore, be expected that after establishment of the equilibrium between exogenous and endogenous mRNAs, also 90% of the injected message is present in the cytosol and only 10% in the polyribosomes, as is the case with endogenous message. If the injected mRNA is diluted by the total pool of endogenous mRNAs, it can only be translated with 10% of its maximal efficiency. The actual efficiency of

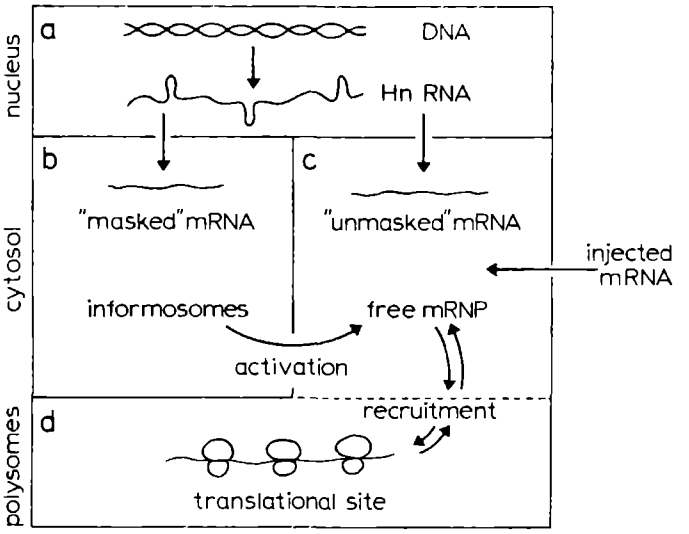



fig. 1: Compartmentation of the mRNA pool in oocytes.

translation of exogenous mRNAs (table 1) seems to be much higher. For example, under conditions, in which 14 S $\alpha A_2$-crystallin mRNA constitutes only 0.4% of the total poly(A) containing mRNA present in the oocyte, 2.5 % of the protein synthesized by the oocyte is $\alpha A_2$-crystallin (table 1, nr. 5). Apparently only a small part of the oocyte mRNA (in compartment C, fig. 1) is competing with the injected mRNA, whereas the rest of the endogenous mRNA is not competing for the translational apparatus, presumably because it is "masked" by some factor (protein or nucleic acid) present in the informosomes (compartment B, fig. 1). The efficiency of translation of exogenous mRNAs varies considerably between batches of oocytes isolated from different frogs (Gurdon et al., 1971; Asselbergs et al., 1978f, 1979d), a phenomenon most likely caused by a variation in the size of the mRNA pool available for translation (compartment C, fig. 1).

Two models for the regulation of mRNA translation in oocytes

Whereas at the moment the identity of the factor (or factors) limiting and, therefore, regulating mRNA translation in oocytes is unknown, four properties of it are obvious: (1) there is no specificity towards different messenger RNA species (Laskey et al., 1977; Asselbergs et al., 1979d); (2) the limiting factors are contained in polyribosomes (Laskey et al., 1977); (3) factors are exchangeable between mRNAs, allowing injected messengers to displace endogenous mRNAs. (4) The number of mRNA molecules translated is regulated by the factors rather than the efficiency of translation of the translated message. (Lingrel and Woodland, 1974). Two viable hypotheses have been put forward concerning the role of the regulating factor(s). First, factors binding to the messenger might 'recruite' a fixed number of

mRNAs for translation (i.e. for transfer from compartment C to D (fig. 1)). (Lingrel and Woodland, 1974). In the second hypothesis the recruitment function is given to the (initiating) ribosomes themselves (Laskey et al., 1977) and ribosomal loading is thought to increase the probability of binding new ribosomes to the mRNA. If this is the case, the mRNA would remain loaded even if initiation is limiting translation. The latter model predicts, that mRNAs engaged in protein synthesis have an advantage in competition over yet untranslated mRNAs. This has indeed been demonstrated (Asselbergs et al., 1979d).

The recruitment of microinjected mRNA appears to be a rather slow process perhaps hampered by physical barriers or diffusion rate, since maximal translation is only observed several hours after injection (Gurdon et al., 1971; Asselbergs et al., 1978f, 1979d). As the injected mRNAs gradually become incorporated into the protein synthesizing machinery of the oocyte (compartment D, fig. 1), they also gradually become less sensitive to competitive inhibition by a second mRNA injected afterwards. Finally after several hours the injected mRNAs are equally sensitive to competition as the oocyte endogenous mRNAs. (Asselbergs et al., 1979d). Just like the endogenous mRNAs, they are then also insensitive to inhibition by poly(A,G,U), a polyribonucleotide which strongly inhibits translation of exogenous mRNA, when coinjected with it (Woodland and Ayers, 1974).

Is feed-back regulation operative in protein synthesis?

Immunoglobulin G consists of two light chains (L) and two heavy chains (H). The intracellular pool of $H_2L_2$ varies depending on the culture conditions of the immunoglobulin producing myeloma cells (Stevens and Williamson, 1973c). At high intracellular $H_2L_2$ concentrations

a specific repression of H-chain synthesis has been observed, suggesting a kind of end-product control of immunoglobulin synthesis. In Xenopus oocytes injected with myeloma polyribosomes co-injected $H_2L_2$ also depressed H-chain synthesis (Stevens and Williamson, 1973b). This effect was attributed to a specific inhibition of translation of H-chain mRNA by $H_2L_2$. (A specific affinity between H-chain mRNA and $H_2L_2$ could initially be demonstrated in vitro (Stevens and Williamson, 1973 b,c), but this specific affinity could no longer be demonstrated in later experiments (Stevens and Williamson, 1975)).

In hemoglobin biosynthesis free α- and β-globin are each thought to inhibit their own synthesis (Adams, 197 ; Blum, Kneip and Schapira, 1972; Blum, Maleknia, Schapira, Kneip and Reibel, 1969; Blum, Maleknia, Schapira, Kneip and Benrubi, 1970; Schaeffer, Trostle and Evans, 1969). Purified rabbit α- and β-globin polypeptides indeed inhibits globin synthesis in oocytes, but not endogenous protein synthesis, when injected together with the mRNA in a 100-fold molar excess over the mRNA (Asselbergs, unpublished). In a similar type of experiment translation of $\alpha A_2$-crystallin mRNA was not inhibited by co-injected α-crystallin or α-crystallin subunits (Asselbergs, unpublished).

Influence of hemin on globin synthesis in oocytes

When reticulocyte lysates are incubated without added hemin, protein synthesis proceeds for only a few minutes and then stops abruptly. With added hemin the translational rate is maintained for hours, because hemin prevents the formation of an inhibitor of polypeptide initiation from an inactive pro-inhibitor (review, Revel and Groner, 1978). The inhibitor reduces the amount of (met-$tRNA_f$.40S), which leads to a decreased synthesis of both globin and non-globin peptides. In absence

of hemin α-globin synthesis in the cell-free system is depressed more than β-globin synthesis probably due to the lower intrinsic rate of ribosome binding of α-globin mRNA (Lodish, 1974). Addition of hemin will restore the equality of α- and β-globin synthesis and has been shown to promote protein synthesis in other cell-free systems (Princen, van Eekelen, Asselbergs and van Venrooij, 1979) as well.

Hemin injected into oocytes of Xenopus laevis (Giglioni et al., 1973; Asselbergs unpublished) or Bufo marinus does not change the overall rate of protein synthesis. In Bufo specific depression of two polypeptides was observed. However, hemin specifically stimulates the translation of rabbit (Lane et al., 1974; Giglioni et al., 1973; Princen et al., 1979) and mouse (Lane et al., 1974) α-globin mRNA in Xenopus oocytes, but not that of the corresponding β-globin mRNAs. When human globin mRNA, isolated from patients with sickle cell anemia is injected together with hemin translation of both α- and $\beta_s$-mRNA was stimulated, but α- more than $\beta_s$-mRNA (Maniatis, Ramirez, Cann, Marks and Bank, 1976). Stimulation of α-globin synthesis was also observed, when hemin is injected a long time after the mRNA (Lane et al., 1974; Giglioni et al., 1973) and when the mRNA was injected in the form of an mRNP (Princen et al., 1979). In the absence of hemin about 4-5 times as much β-globin as α-globin is made in Xenopus oocytes injected with polysomal globin mRNA. A relative excess of α-globin synthesis was observed in oocytes of Bufo marinus (May and Glenn, 1974). In Xenopus oocytes translation of α-globin mRNA containing, non-polysomal mRNP particles from rabbit reticulocytes (Princen et al., 1979) as well as of the messenger RNA isolated from these particles (Giglioni et al., 1973; Princen et al., 1979; Huez, Marbaix, Gallwitz, Weinberg, Devos, Hubert and Cleuter, 1978) was stimulated by hemin. Apparently some restriction of the

translation of α-globin mRNA existing in Xenopus oocytes is releaved by hemin. This restriction can apparently be overcome also with the aid of a component of reticulocyte polyribosomes, since injected polyribosomes synthesize α- and β-globin in a 1:1 ratio in the absence of hemin (Gurdon et al., 1973). The restriction of α-globin synthesis in Xenopus oocytes does not lead to a drastic reduction of the α-globin synthesizing polyribosomes (Lingrel and Woodland, 1974).

Control of translation of globin mRNA in embryonic cells

When rabbit globin mRNA is injected into fertilized eggs of Xenopus laevis, a sudden increase in the ratio α- to β-globin synthesized is observed at the time of gastrulation (Lane et al., 1974), which is maintained during further development (followed up to the stage, when the tadpoles start swimming). This 'spontaneous' stimulation of the α-globin synthesis is not observed, when mouse globin mRNA is injected. The action of the embryo factor is, therefore, distinct from the action of hemin, which equally stimulates the synthesis of mouse and rabbit α-globin (Lane et al., 1974). Hemin and the embryo factor do not seem to have an additive effect on rabbit α-globin synthesis in Xenopus neurulae.

Globin mRNA injected into fertilized Xenopus eggs seems to be extremely stable (Gurdon and Woodland, 1974; Lane et al., 1974; Woodland, Gurdon and Lingrel, 1974). Globin synthesis is constant during embryonic development, although it becomes increasingly obscured by the rising synthesis of endogenous proteins (Gurdon and Woodland, 1974). Apparently, the established globin message is not inactivated, when stored endogenous mRNAs and ribosomes are mobilized into action.

Stability of injected mRNAs in oocytes

In contrast to the cells of the developing embryo, full-grown oocytes are cells with a steady state metabolism. Cultured in vitro in a non-nutrient medium, oocytes remain metabolically active for about two weeks, occasionally up to a month, during which period no major changes are observed in, for example, the pattern of synthesized polypeptides. These properties make oocytes a very useful system to study regulatory processes that influence the stability of mRNA.

Rabbit and mouse globin mRNA micro-injected into Xenopus oocytes fully maintained their initial translational activity (normalized in respect to the endogenous protein synthesis) during the culturing of the oocyte (Gurdon et al., 1973) which, in this study, varied from 1 to 3 weeks between batches of oocytes from different frogs. α- and β-globin mRNA appeared to be equally stable. During culture in vitro a gradual decrease in the rate of incorporation of radioactive amino acid into both globin and endogenous proteins was observed. However, globin mRNA injected into oocytes which already had been cultured in vitro for several days, was translated as efficient as mRNA, which already had been translated in the oocytes for the entire culture period (Gurdon et al., 1973). The decreased incorporation into globin was, therefore, thought to arise from a change in the metabolism of the oocyte (presumably an increased intracellular amino acid concentration (Bravo, Salazar and Allende, 1976)) and not to degradation of the mRNA. Such a remarkable functional stability has also been demonstrated for other poly(A)-containing mRNA species, notably those encoding chicken ovalbumin (Chan et al., 1976), calf $\alpha A_2$-crystallin (Asselbergs et al.,

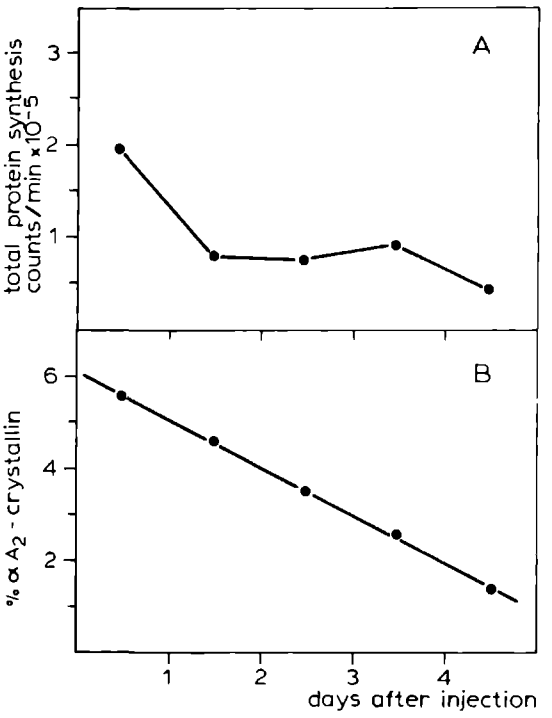



fig. 2: Occasional instability of $\alpha A_2$-crystallin mRNA in oocytes

Batches of 5 oocytes were injected with 5 ng poly(A) containing calf lens mRNA, incubated in modified Barth's medium with 0.5 mCi/ml for 20 h periods at various times after injection and the translation products were quantitated by immunoprecipitation with rabbit antiserum against $\alpha A_2$-crystallin as described in III-2 (Asselbergs, van Venrooij and Bloemendal, 1978). The middle of the labeling period is indicated.
(A) Total incorporation of radioactivity into protein per oocyte.
(B) Incorporation into $\alpha A_2$-crystallin is expressed as a percentage of (A). This percentage can be regarded as a measure for the translational activity of the 14S $\alpha A_2$-crystallin mRNA, which is generally a stable messenger RNA (cf. III-2; Asselbergs, van Venrooij and Bloemendal, 1978). The mRNAs encoding $\beta$- and $\gamma$-crystallin were equally unstable in this batch of oocytes (data not shown).

1978f, Xenopus serum albumin and vitellogenin (Berridge and Lane, 1976) human placental lactogen (Mous, Peters, Van Belleghem and Rombauts, 1977, 1979) and the RNA isolated from Rauscher murine leukemia virus (Asselbergs, Salden and Bloemendal, 1979c). However, it sometimes happens that in certain batches of quite normally looking oocytes, the injected mRNA is functionally unstable. In those exceptional cases (Gurdon et al., 1973; cf. fig. 2) the inactivation seems to follow linear kinetics.

In the experiments mentioned above, the functional stability of the injected mRNA was measured. This parameter may be different from the physical stability, for example, because not all mRNA molecules are translated. Allende, Allende and Firtel (1974) studied the fate of $^{32}P$-labeled poly(A) containing mRNA from the slime mold, Dictyostelium discoideum, and from vesicular stomatitis virus after injection in oocytes. They found, that the mRNA decay followed biphasic kinetics. About 60% of the mRNA was degraded in the first 6h, whereas the remaining 30-40% was stable for at least 22 h. The stable fraction was apparently protected against nucleolytic degradation by means of an association with ribosomes, since it was not found when puromycine was present in the incubation medium. On the other hand, rabbit globin mRNA, quantitated by molecular hybridisation, was not destabilized by puromycine (Huez et al., 1977a).

In a comment on some of the experiments above, Hunt (1975) suggested to use iodine labeled globin mRNA to study the stability of mRNA in oocytes. Such experiments were performed by the author, in collaboration with Dr. L.H. Cohen. It was found that the iodinated mRNA quickly decayed in oocytes (fig. 3). It is possible that the mRNA

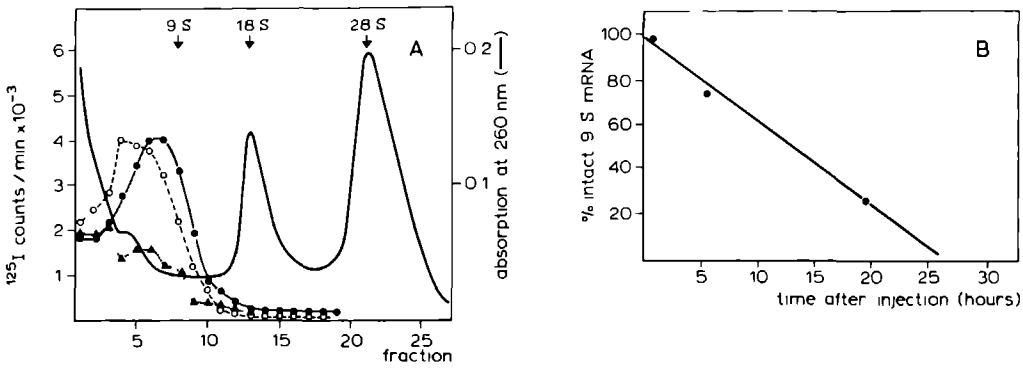



Fig. 3: Decay of iodine labeled globin mRNA in oocytes

Rabbit globin mRNA (9 S), purified by affinity chromatography on oligo(dT) cellulose and sucrose gradient centrifugation (13), was labeled with iodine-125 (spec. act. 11-17 mCi/µg) as described by Prensky (1976) to a specific activity of $10^7$ cpm/µg mRNA. Thus, each globin mRNA molecule carries 2-3 iodine atoms. After iodination the mRNA was rechromatographed on oligo(dT)cellulose and cosedimented with unlabeled globin mRNA during sucrose gradient centrifugation.

(A) Batches of 10 oocytes were injected with 50 nl containing 0.5 ng iodinated globin mRNA (5 x $10^3$ counts/min), oocytes were incubated for 1 h (●—●), 6 h (o---o) and 18 h (▲...▲), homogenized in 10 mM Tris/HCl ph = 7.4 containing 5 mM ethylene diaminotetra-acetic acid, 0.5% sodium dodecylsulphate and carrier ribosomal RNA from rabbit reticulocytes, and the lysates were stored at -80°C. Lysates were layered on 15-35% (w/w) isokinetic sucrose gradients for the IEC SB 283 rotor containing 10 mM Tris/HCl pH = 7.4. The gradients were spun for 22 h at 5°C and pumped through the flow cell (path length 0.25 cm) of a Gilford spectrofotometer. Total radioactivity of 0.3 ml fractions was measured using Aquasol (New England Nuclear) as a scintillant.

(B) % of the labeled mRNA cosedimenting with intact (uninjected) globin mRNA (fractions 7-9).

was functionally impaired due to the specific removal during the iodination procedure (Prensky, 1976) of an oligonucleotide, which is part of the ribosome binding site of the mRNA (Legon, Robertson and Prensky, 1976). After 4 h incubation in oocytes only 6% of the radioactivity was found in polyribosomes, in contrast to 20-40% after 40 min. in a reticulocyte lysate indicating that the iodinated message was impaired in a function primarily needed for survival in vivo (data not shown).

Role of the poly(A) sequence in the stability of mRNA in oocytes

Most eukaryotic mRNA species carry a stretch of poly(A), about 20-100 residues long, at their 3' OH end. It seems, that the poly(A) segments become shorter with age, as has clearly been demonstrated for mouse (Gorski, Morrison, Merkel and Lingrel, 1975; Merkel, Kwan and Lingrel, 1976) and rabbit globin mRNAs (Nokin, Huez, Marbaix, Burny and Chantrenne, 1976; Nokin, Burny, Huez and Marbaix, 1976). Since some mRNAs, for example histone mRNAs (Adesnik and Darnell, 1972; Greenberg and Perry, 1972), generally do not contain poly(A), it seems unlikely that the presence of poly(A) is required for translation. Globin mRNA from which the poly(A) had been removed (Bard, Efron, Marcus and Perry, 1974; Sippel, Stavrianiopoulos, Schutz and Feigelson, 1974; Soreq, Nudel, Salomon, Revel and Littauer, 1974; Williamson, Crossley and Humphries, 1974), or Encephalomyocarditis viral RNA selected for a low poly(A) content (Hruby, 1978) were initially translated in cell-free systems as efficiently as their polyadenylated counterparts. Only, during prolonged incubation translation of the poly(A)-free mRNAs was much less efficient.

When injected into oocytes, the initial rate of translation of

poly(A)-free rabbit globin mRNA was close to that of complete mRNA. However, after an incubation of two days, the rate of globin synthesis directed by poly(A)-free mRNA was more than 10-fold lower than in the case of native mRNA (Huez et al., 1974). Poly(A)-poor fractions of human globin mRNA also had a lower functional stability in oocytes than poly(A)-rich fractions (Maniatis et al., 1976). Re-addition of poly(A)-tails could restore the stability of deadenylated rabbit globin mRNA (Huez et al., 1975). From experiments in which globin mRNA preparations with well-defined poly(A) lengths were injected into Xenopus oocytes, it was concluded that a minimum length of about 30 adenyl residues was needed to confer complete functional stability to the mRNA. (Nudel, Soreq, Littauer, LeClercq, Hubert, and Chantrenne, 1976). Messenger RNA with a poly(A) segment of less than 15 adenines was as unstable as mRNA totally devoid of poly(A). Messenger RNAs with poly(A) segments between 15 and 30 residues long mRNAs had an intermediate stability. When the injected globin mRNA was quantitated by molecular hybridisation with cDNA prepared with reverse transcriptase, it was found, that 85% of the poly(A)-free globin mRNA had been degraded two days after injection, whereas the native mRNA was almost completely preserved (Marbaix et al., 1975). A poly(A) stretch of sufficient length is, therefore, required to ensure the physical stability of injected globin mRNA.

Hemin which promotes translation of α-globin mRNA in oocytes (above), also accelerated degradation of poly(A)-free α-globin mRNA. This finding suggests, that a poly(A)-free messenger RNA must be translated for its physiological degradation to occur. (Huez et al., 1977a).

Micro-injected histone mRNAs, which lack a poly(A) segment (Huez et al., 1977b, 1978), were found to be functionally unstable in oocytes. The histone mRNA could be stabilized by the addition of a poly(A) stretch (Huez et al., 1978). In somatic cells histone synthesis occurs only parallel to DNA synthesis and the histone mRNA is quickly degraded at the end of the S-phase of the cell cycle (Perry and Kelley, 1973). In oocytes continuous histone synthesis occurs in the absence of DNA synthesis. In addition oocytes must contain a considerable amount of stable untranslated histone message, since histone synthesis increases 20 to 100-fold during maturation, even in the absence of RNA synthesis (Adamson and Woodland, 1977). It is reasonable to assume that the remarkable stability of the oocyte histone mRNA is a consequence of the presence of a poly(A) segment, which has been shown to be present on histone mRNA of amphibian oocytes (Levinson and Marai, 1976; Ruderman and Pardue, 1978).

Despite the presence of a poly(A) segment on interferon mRNA of human fibroblasts, the rate of interferon accumulation in oocytes gradually decreases after injection of the mRNA. Removal of the poly(A) segment from the interferon mRNA does not affect the pattern of interferon accumulation described above (Seghal, Soreq and Tamm, 1978). The translational stability of Mengo virus RNA (Seghal et al., 1978) is also not affected by removal of the poly(A) segment. Furthermore, the translational stability of hepatic $\alpha_{2u}$ globulin mRNA was not influenced by the length of the poly(A) stretch (Deshpande and Chatterjee, 1979). It thus appears that other factors besides the presence and the length of the poly(A)-tail influence the functional stability of (some) mRNAs in oocytes.

Role of the 'cap' in oocytes

Most eukaryotic mRNAs contain a blocked, methylated 5'-terminal 'cap', $m^7G(5')ppp(5')X$- (reviews, Shatkin, 1976; Filipowicz, 1978). The presence of the cap facilitates mRNA translation at the level of the initiation of protein synthesis in many cell-free systems. Apart from this role in translation, the cap also protects the mRNA from exonucleolytic degradation from the 5'-end both in cell-free systems derived from wheat germ (Shimotohno, Kodama, Hashimoto and Miura, 1977) and L-cells (Furuichi, LaFiandra and Shatkin, 1977) as well as in intact Xenopus oocytes (Furuichi et al., 1977). In oocytes 60% of the capped ($^{32}$-P labeled) reovirus mRNA, with 5'-end $m^7G(5')pppG^m$, was degraded within 24 hours. This instability of the control mRNA is perhaps a consequence of the absence of poly(A) in reovirus mRNA (Stoltzfus, Shatkin and Banerjee, 1974; Both, Lavi and Shatkin, 1975). Blocked but unmethylated reovirus mRNA ending in G(5')ppp(5')G, was as unstable as capped mRNA. A considerable part of the blocked 5' termini were methylated in the oocyte to $m^7G(5')ppp(5')G$ and $m^7G(5')ppp(5')G^m$. Unblocked mRNAs, ending in ppp(5')G and $ppp(5')G^m$, were degraded much faster than blocked mRNAs. Eight hours after injection 55% of the blocked and capped mRNAs was still intact, whereas only 20-25% of the radioactive mRNA with unblocked 5' termini was still acid precipitable. Only 10% of the unblocked mRNAs remained after 24 h.

In another type of experiment, translation of rabbit globin mRNA from which the cap had been removed by chemical or enzymatical methods, in oocytes was reduced to less than 5% (Lockard and Lingrel, 1978). RNA of the Encephalomyocarditis virus, one of those exceptional eukaryotic mRNAs which is not capped, was still translated 2 days after injection

in oocytes (Laskey, Gurdon and Crawford, 1972). Translation of exogenous and endogenous mRNA was not inhibited after injection of large amounts of cap-analogues, $m^7G(5')p$ and $m^7G(5')ppp(5')G$ (section III-4: Asselbergs, Peters, van Venrooij, and Bloemendal, 1978b) whereas cap-analogues strongly inhibited translation of these mRNAs in vitro (Asselbergs et al., 1978 c-e; Darnborough and Ford, 1976). It is, therefore, at present unclear whether the cap, apart from its protective role, plays also an essential role in the translation of capped mRNAs in oocytes. However, the observation that periodate oxidized rabbit globin mRNA, in which the chemically modified cap probably still protects the mRNA against 5' exonucleolytic degradation, is not translatable in oocytes (Lockard and Lane, 1978), is perhaps an indication that the cap is essential for proper translation of capped mRNAs in vivo.

Concluding remarks

Micro-injection experiments with oocytes, as described above, have already revealed the first glimpses of the complex pattern of regulatory mechanisms operative during mRNA translation. However, a number of provocative questions remain. For example: (1) What is the nature of the polysomal factors limiting protein synthesis? (2) Is untranslated exogenous mRNA as stable as translated mRNA? (All stability experiments so far have been performed at low mRNA concentrations, when, as judged from the translational efficiency, most of the input mRNA is translated. If a higher mRNA concentration is injected some of the mRNA molecules are not translated and perhaps unstable.) (3) Through what kind of mechanism are informosomal mRNAs kept inactive and is the activation to some extent mRNA specific?

Table 3A: Messenger RNAs translated in amphibian oocytes-Mammals

| Protein | organ/cell type | species | references |
|---|---|---|---|
| a. globin | reticulocyte | rabbit | Lane et al. (1971); Gurdon et al. (1971); Marbaix and Lane (1972); Brachet et al., (1973); May and Glenn (1974); Gurdon et al., (1973); Marbaix and Gurdon, (1972). |
| | | mouse | Gurdon et al., (1973); Marbaix and Gurdon, (1972). |
| | | man | Maniatis et al., (1976). |
| | leukemic cell | mouse | Ostertag et al., (1973). |
| b. crystallin | eye lens | calf | Asselbergs et al., (1978 a,b,f)(1979 a,d) Berns et al., (1972). |
| c. uteroglobin | uterus | rabbit | Beato and Nieto, (1976); Beato and Rungger, (1975). |
| d. milk protein | mammary gland | guinea pig | Campbell et al., (1973). |
| e. thyroglobulin | thyroid gland | horse | Vassart et al., (1973). |
| | | cattle | DeNayer et al., (1974), Vassart et al.,(1974)(1975 a and b) |
| f. myelin basic protein | brain | rat | Lim et al., (1974 a,b) White et al., (1975). |
| g. immunoglobulin G | plasmacytoma | mouse | Mach et al., (1973); Jilka et al., (1977)(1979); Stevens and Williamson, (1972) (1973 a,b), Smith et al. (1973) |
| | spleen | mouse | Deacon and Erbringer, (1977) |
| | | rat | Stevens et al. (1975) |
| h. collagen | fibroblast | man | Lane and Knowland, (1975). |
| i. lactogen | placenta | woman | Mous et al., (1976)(1977b)(1979). |
| j. tyrosine amino-transferase | liver | rat | Nickol et al., (1976). |
| k. tubulin | brain | mouse | van der Saag, (1975). |
| l. steroid binding protein | prostrate | rat | Mous et al., (1977a) |
| m. interferon | fibroblast | man | Cavalieri et al., (1977 a,b), Raj and Pitha, (1977); Reynolds et al., (1975); Seghal et al., (1977). |
| | leukocyte | man | Seghal et al., (1977). |
| | fibroblast | mouse | Lebleu et al., (1978). |

Table 3A: Messenger RNAs translated in amphibian oocytes--(Continued)

| Protein | organ/cell type | species | references |
|---|---|---|---|
| n. insulin | insulinoma | man | Yip et al., (1975), Rapaport et al., (1978). |
| o. β-glucuronidase | kidney | mouse | Labarca and Paigen, (1977). |
| p. histone | HeLa-cell | man | Huez et al., (1977b) (1978) |
| q. unidentified proteins | HeLa-cell | man | Gurdon et al., (1976). |
| r. $\alpha_{2u}$ globulin | liver | rat | Desphande and Chatterjee, (1979). |
| s. thyroglobulin (α-subunit) | thyrotropic pituitary tumor | mouse | Kourides and Weintraub, (1979). |
| BIRDS | | | |
| globin | reticulocyte | duck | Lane et al., (1973). |
| ovalbumin | oviduct | chicken | Chan et al., (1976). |
| vitellogenin | liver | chicken | Gruber, M. et al., (1976). |
| AMPHIBIA | | | |
| collagen | tadpole tail | Xenopus | Rollins and Flickinger, (1972), Flickinger and Rollings, (1974). |
| vitellogenin | liver | Xenopus | Berridge and Lane, (1976) Lanclos and Hamilton, (1975). Wiggins et al.,(1978). |
| albumin | liver | Xenopus | Berridge and Lane (1976), Zehavi-Willner and Lane (1977). |
| FISH | | | |
| insulin | pancreatic islets | cod | Yip et al., (1975) |
| freezing point depressing protein | liver | American winter flounder | Hew and Yip, (1976). |
| protamin | testis | trout | Gedamu et al., (1978). |

Table 3A: Messenger RNAs translated in amphibian oocytes--(Continued)

| Protein | organ/cell type | species | references |
|---|---|---|---|
| INSECTS | | | |
| mitellin | venom gland | honeybee | Kindas-Mugge et al., (1974), Kreil et al., (1977) Lane (1976). |
| hormone induced proteins | flight muscle | cricket | Moser et al., (1975). |
| | $K_c$-cells | Drosophila | Moser et al., (1975). |
| Echinodermata | | | |
| histone | embryo | Lytechinus pictus | Huez et al., (1977b). |
| Higher plants z | | | |
| flavone synthetase | suspension culture cells | Petroselinum hortense (Hoffm.) | Schroeder et al., (1977). |
| phenylalanine ammonia lyase (PAL) | suspension culture cells | Petroselinum hortense (Hoffm.) | Schroeder et al., (1977). |
| YEASTS | | | |
| mitochondrial (?) proteins | | Sacharomyces | Eggit and Scragg (1975) |

Table 3B: Messenger RNAs translated in unfertilized amphibian eggs

| Protein | organ/cell type | species | references |
|---|---|---|---|
| MAMMALS | | | |
| a. globin | reticulocyte | rabbit | Gurdon et al., (1971)(1973), Moar et al., (1971). |
| b. immunoglobulin G | plasmacytoma | mouse | Stevens and Williamson, (1973b). |
| c. calcitonin | tumor cell | man | van der Donk et al., (1976). |
| d. hormone precursors | several tumors | | Lips et al., (1978). |
| e. immunoglobulin E | plasmacytoma | mouse | van der Donk et al., (1978). |
| f. immunoglobulins G,M,A | colostrum | pig | Kortbeek-Jacobs et al., (1978). |
| HIGHER PLANTS | | | |
| f. incompatibility protein | flower style | Petunia | van der Donk et al., (1975). |

Table 3C: Messenger RNAs translated in developing amphibian embryos

| | | | |
|---|---|---|---|
| MAMMALS | | | |
| a. globin | reticulocyte | rabbit | Brachet et al., (1973); Froehlich et al., (1977), Gurdon et al., (1974 Lane et al., (1974); Woodland et al., (1974). |
| | | mouse | Gurdon et al., (1974); Lane et al., (1974). |

Table 4: Translation of viral (messenger) RNAs in amphibian oocytes--Animal viruses

| Virus | mRNA type | species | references |
|---|---|---|---|
| Picornaviruses | | | |
| Encephalomyocarditis virus | genomic RNA | mouse | Laskey et al., (1972) (1977). |
| Retroviruses | | | |
| Avian myeloblastosis virus | genomic RNA | chicken | Ghysdael et al., (1977) a,b); Salden et al., (1976a). |
| Avian sarcoma virus | genomic RNA | chicken | Katz et al., (1979). |
| Rauscher leukemia virus | genomic RNA | mouse | Asselbergs et al., (1979 c) Salden et al., (1976 b). |
| | cellular mRNA (genome size and subgenomic mRNAs) | | van Zaane et al., (1977). |
| Abelson leukemia virus | genomic RNA | mouse | Reynolds et al., (1978). |
| Bovine leukemia virus | genomic RNA | cattle | Ghysdael et al., (1979), Burny et al. (1978). |
| Mammary tumor virus | genomic RNA | mouse | Nusse et al., (1978). |
| Adenoviruses | | | |
| Adenovirus 5 | late mRNA | man | DeRobertis et al., (1977) |
| Plant viruses | | | |
| Tobacco mosaic virus (undivided genome) | genomic RNA subenomic mRNA | tobacco | Knowland (1974). Hunter et al., (1976). |
| Alfalfa mosaic virus (tripartite genome) | RNAs 1,3,4 | alfalfa | Rutgers (1977) Rutgers et al., (1976) Van Vloten-Doting (1977) |
| Brome mosaic virus (tripartite genome) | RNAs 1,2,3,4 | brome grass | Rutgers (1977) Semancik, et al., (1977) |
| Cucumber mosaic virus (tripartite genome) | RNAs 3,4 | cucumber | Schwinghamer and Symons (1977). |

## References


1. Adams, J.G. (1975) Annals N.Y. Acad. Sci. , 232-241.

2. Adamson, E.D. and Woodland, H.R. (1977) Dev. Biol. 57, 136-149.

3. Adesnik, M. and Darnell, J.E. (1972) J. Mol. Biol. 67, 397-406.

4. Allende, C.C., Allende, J.E. and Firtel, R.A. (1974) Cell 2, 186-196.

5. Asselbergs, F.A.M., Koopmans, M., van Venrooij, W.J. and Bloemendal, H. (1978a) Eur. J. Biochem. 91, 65-72 (section IV-2)

6. Asselbergs, F.A.M., Koopmans, M., van Venrooij, W.J. and Bloemendal, H. (1979a) Exp. Eye Res., in press.

7. Asselbergs, F.A.M., Meulenberg, P.M.M., van Venrooij and Bloemendal, H. (1979b) submitted for publication.

8. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij and Bloemendal, H. (1978b) FEBS Lett. 94, 195-198. (section III-4).

9. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij, and Bloemendal, H. (1978c) Biochim. Biophys. Acta 520, 577-587.

10. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij and Bloemendal, H. (1978d) Eur. J. Biochem. 88, 483-488.

11. Asselbergs, F.A.M., Peters, W.H.M., van Venrooij, W.J. and Bloemendal, H. (1978e) Mol. Biol. Rep. 4, 177-180.

12. Asselbergs, F.A.M., Salden, M.H.L. and Bloemendal, H. (1979c) submitted for publication (section V-3).

13. Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H. (1978f) Eur. J. Biochem. 87, 517-524 (section III-2).

14. Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H. (1979d) Eur. J. Biochem. 94, 249-254 (section III-3).

15. Bard, E., Efron, D., Marcus, A. and Perry, R.P. (1974) Cell 1, 101-106.

16. Beato, M. and Nieto, A. (1976) Eur. J. Biochem. 64, 15-25.

17. Beato, M. and Rungger, D. (1975) FEBS Lett 59, 305-309.

18. Berns, A.J.M., van Kraaikamp, M., Bloemendal, H. and Lane, C.D. (1972) Proc. Natl. Acad. Sci. USA 69, 1606-1609.

19. Berridge, M.V. and Lane, C.D. (1976) Cell 8, 283-292.

20. Blum, N., Kneif, B. and Shapira, G. (1972) Biochimie 54, 1121-1128.

21. Blum, N., Maleknia, N., Shapira, G., Kneip, B. and Reibel, L. (1969) Biochim. Biophys. Acta. 179, 448-463.

22. Blum, N., Maleknia, M., Shapira, G., Kneip, B. and Benrubi, M. (1970) Biochim. Biophys. Acta. 199, 236-247.

23. Both, G.W., Lavi, S. and Shatkin, A.J. (1975) Cell 4, 173-180

24. Brachet, J., Huez, G. and Hubert, E. (1973) Proc. Natl. Acad. Sci. USA 70, 543-547.

25. Bravo, R., Salazar, I. and Allende, J.E. (1976) Exptl. Cell Res. 103, 169-174.

26. Burny, A., Bex, F. Chantrenne, H., Cleuter, Y., Dekegel, D., Ghysdael, J., Kettmann, R., Leclercg, M., Leunen, J., Mammerickxs, M. and Portetelle, D. (1978) Adv. in Cancer Res., Vol. 28 (G. Klein and S. Weinhouse, eds). Acad. Press, New York, pp. 252-312.

27. Cabada, M.D., Darnborough, C., Ford, P.J. and Turner, P.C. (1977) Dev. Bio. 57, 427-439.

28. Campbell, P.N., McJlraevy, D. and Tarin, D. (1973) Biochem. J. 134, 345-347.

29. Cairns, E., Gschwender, H.K., Primke, M., Yamakawa, M., Traub, P. and Schweiger, H.G. (1978) Proc. Natl. Acad. Sci. USA 75, 5557-5559.

30. Cavalieri, R.L., Havell, E.A., Vilchek, J. and Pestka, S. (1977a) Proc. Natl. Acad. Sci. USA 74, 3287-3291.

31. Cavalieri, R.L., Havell, E.A., Vilckek, J. and Pestka, S. (1977b) Proc. Natl. Acad. Sci. USA 74, 4415-4419.

32. Chan, L., Kohler, P.O. and O'Malley, B.W. (1976) J. Clin. Invest. 57, 576-585.

33. Cox, R.A., Ford, P.J. and Pratt, H. (1970) Biochem. J. 119, 161-164.

33a. Crick, F. (1979) Science 204, 264-271.

34. Darnborough, C. and Ford, P.J. (1976) Dev. Biol. 50, 285-301.

35. Davidson, H. (1976) Gene activity in development 2nd edition, Acad. Press., New York.

36. Deacon, N.H. and Erbringer, A. (1977) Proc. Aust. Biochem. Soc. 10, 89.

37. Deacon, N.J. and Erbringer, A. (1977) FEBS Lett 79, 191-194.

38. DeNayer, P. Durieux, B., Nyssen-Gautier, F. and DeVisscher, M. (1974) Life Sciences 14, 625-630.

39. DeRobertis, E.M., Partington, G.A., Longthorne, R.F. and Gurdon, J.B. (1977) J. Embryol. Exp. Morph. 40, 199-214.

40. Deshpande, A.K. and Chatterjee, B. (1979)Fed. Proc. 38, 782.

41. Dimitriados, G.J. (1978) Nature 274, 923-924.

42. Eggit, M. and Scragg, A.H. (1975) Biochem. J. 149, 507-512.

43. Flickinger, R.A. and Rollins, J.W. (1974) Exptl. Cell Res. 89, 402-404.

44. Froehlich, J.P., Browder, L.W. and Schultz, G.A. (1977) Dev. Biol. 56, 356-371.

45. Filipowicz, W. (1978) FEBS Lett 96, 1-11.

46. Furuichi, Y., LaFiandra, A. and Shatkin, A.J. (1977) Nature 266, 235-239.

47. Gatica, M., Tarrago, A., Allende, C.C. and Allende, J.E. (1977) Nature 256, 675-677.

48. Gedamu, L., Dixon, G.H. and Gurdon, J.B. (1978) Exptl. Cell Res. 117, 325-334.

49. Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D.P., Cleuter, Y., Huez, G., Marbaix, G., Portetelle, D. and Chantrenne, H. (1977) Biochem. Soc. Trans. 5, 950-953.

50. Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D.P., Burny, A., Cleuter, Y., Huez, G., Kettmann, R., Marbaix, G., Portetelle, D., and Chantrenne, H. (1977) Proc. Natl. Acad. Sci. USA 74, 3230-3234.

51. Ghysdael, J. Kettmann, R. and Burny, A. (1979) J. Virol. 29, 1087-1098.

52. Giglioni, B., Gianni, A.M., Comi, P., Ottolenghi, S. and Rungger, D. (1973)

53. Glenn, A.R. and May, B.K. (1975) Aust. J. Biol. Sci. 28, 749-751.

54. Gorski, J., Morrison, M.R., Merkel, C.G. and Lingrel, J. (1975) Nature 253, 749-751.

55. Graessmann, A. and Graessmann, M. (1971) Hoppe Zeylers, Z. Physiol. Chem. 35, 527-532.

56. Greenberg, J. and Perry, R.P. (1972) J. Mol. Biol. 72, 91-98.

57. Gruber, M. cited in: Campbell P.N. and Blobel, G. (1976) FEBS Lett. 72, 215-226.

58. Gurdon, J.B. (1973) Acta. Endocrin. supp. 80, 225-243.

59. Gurdon, J.B. (1974) Nature 260, 116-120.

60. Gurdon, J.B., DeRobertis, E.M. and Partington, G. (1976) Nature 260, 116-120.

61. Gurdon, J.B., Lane, C.D., Woodland, H.R. and Marbaix, G. (1971) Nature 233, 177-182.

62. Gurdon, J.B., Lingrel, J.B. and Marbaix, G. (1973) J. Mol. Biol. 80, 539-551.

63. Gurdon, J.B. and Woodland, H.R. (1974) Dev. Bio. 39, 125-133.

64. Herrlich, P. and Schweiger, M. (1978) FEBS Lett 87, 1-6.

65. Hew, C.L. and Yip, C. (1976) Biochem. Biophys. Res. Commun. 71, 845-850.

66. Hruby, D.E. (1978) Biochem. Biophys. Res. Commun. 81, 1425-1434.

67. Huez, G., Marbaix, G., Burny, A., Hubert, E., Leclercq, M. Cleuter, Y., Chantrenne, H., Soreq, H. and Littauer, U.Z. (1977) Nature 266, 473-474.

68. Huez, G., Marbaix, G., Gallwitz, D., Weinberg, E., Devos, R., Hubert, E. and Cleuter, Y. (1978) Nature 271, 572-573.

69. Huez, G., Marbaix, G., Hubert, E., Cleuter, Y., Leclercq, M., Chantrenne, H., Devos, R., Soreq, H., Nudel, U. and Littauer, U.Z. (1975) Eur. J. Biochem. 59, 589-592.

70. Huez, G. Marbaix, G., Hubert, E., Leclercq, M., Nudel, U. Soreq, H., Salomonson, R., Lebleu, B., Revel, M. and Littauer, U.Z. (1974) Proc. Natl. Acad. Sci. USA 71, 3134-3146.

71. Huez, G., Marbaix, G., Weinberg, E., Gallwitz, D., Hubert, E. and Cleuter, Y. (1977) Biochem. Soc. Trans. 5, 936-937.

72. Hunt, T. (1975) Nature 253, 308.

73. Hunt, T. Hunter, T. and Munro, A. (1969) J. Mol. Biol. 43, 123-133.

74. Hunter, T., Hunt, T., Knowland, J. and Zimmern, D. (1976) Nature 260, 758-764.

75. Ilan, J. and Ilan, J. (1976) J. Biol. Chem. 251, 5718-5725.

76. Jeffreys, A. and Flavell, R.A. (1977) Cell 12, 1097-1108.

77. Jilka, R.L., Cavalieri, R.L., Yaffe, L. and Pestka, S. (1977) Biochem. Biophys. Res. Commun. 79, 625-630.

77a. Jilka, R.L., Faniletti, P. and Pestka, S. (1979) Arch. Biochem. Biophys. 192, 290-295.

78. Katz, R.A., Maniatis, G.M. and Guntaka, R.V. (1979) Biochem. Biophys. Res. Commun. 86, 447-453.

79. Kindas-Mugge, I., Lane, C.D. and Kreil, G. (1974) J. Mol. Biol. 87, 451-462.

80. Kinniburgh, A.J., Mertz, J.E. and Ross, J.E. (1978) Cell 14, 681-693.

81. Knowland, J. (1974) Genetics 78, 383-394.

82. Kortbeek-Jacobs, N. and van der Donk, H. (1978) J. Immunol. Meth. 24, 195-199.

83. Kourides, I. and Weintraub, B.D. (1979) Proc. Natl. Acad. Sci. USA 76, 298-302.

84. Kozak, H. (1978) Cell 15, 1109-1123.

85. Kreil, G., Suchanek, G. and Kindas-Mugge, J. (1977) Fed. Proc. 36, 2081-2086.

86. Labarca, C. and Paigen, K. (1977) Proc. Natl. Acad. Sci. USA 74, 4462-4465.

87. Lanclos, K.D. and Hamilton, T.H. (1975) Proc. Natl. Acad. Sci. USA 72, 3934-3938.

88. Lane, C.D. (1976) Scientific American 234, 60-71.

89. Lane, C.D., Gregory, C.M., Iazumi, T. and Scherrer, K. (1973) Nature New Biol. 243, 78.

90. Lane, C.D., Gregory, C.M. and Morel, C. (1973) Eur. J. Biochem. 34, 219-229.

91. Lane, C.D., Gurdon, J.B. and Woodland, H.R. (1974) Nature 251, 436-437.

92. Lane, C.D. and Knowland, J. (1975) in Biochemistry of development, Vol 3 (R. Weber, ed) New York, Acad. Press, pp 145-181.

93. Lane, C.D., Marbaix, G. and Gurdon, J.B. (1971) J. Mol. Biol. 61, 73-91.

94. Laskey, R.A., Gurdon, J.B. and Crawford, L.V. (1972) Proc. Natl. Acad. Sci. USA 69, 3665-3669.

95. Laskey, R.A., Mills, A.D., Gurdon, J.B. and Partington, G.A. (1977) Cell 11, 345-351.

96. Lebleu, B., Hubert, E., Content, J., DeWit, L., Brande, I.A. and DeClercq, E. (1978) Biochem. Biophys. Res. Commun. 82, 665-673.

97. Legon, S., Robertson, H.D. and Prensky, W. (1976) J. Mol. Biol. 106, 23-36.

98. Levinson, R.G. and Marai, K.B. (1976) Cell 9, 311-31

99. Lim, L., White, J.O., Hall, C., Berthold, W. and Davison, A. (1974) Biochem. Soc. Trans. 2, 245.

100. Lim, L., White, J.O., Hall, C., Berthold, W and Davison, A. (1974) Biochim. Biophys. Acta. 361, 241-247.

100a. Lingrel, J.B. and Woodland, H.R. (1974) Eur. J. Biochem. 47, 47-56.

101. Lips, C.J.M., van der Donk, J.A., Hackeng, W.H.L. and van der Sluys-Veer, J. (1978) Ned. J. Med. 21, 139-140.

101a. Lockard, R.E. and Lane, C.D. (1978) Nucleic Acids Res. 5, 3237-3248.

102. Lodish, H.F. (1971) J. Biol. Chem. 246, 7131-7138.

103. Lodish, H.F. (1974) Nature 251, 385-388.

104. Lodish, H.F. (1976) Ann Rev. Biochem. 45, 39-72.

105. Lodish, H.F. and Froshauer, S. (1977) J. Biol. Chem. 252, 8804-8811.

106. Mach, B., Faust, C.F., Vassali, P. and Rungger, D. (1973) Mol. Biol. Rep. 1, 3-6.

107. Maniatis, G.M., Ramirez, F., Cann, A., Marks, P. and Bank, A. (1976) J. Clin. Invest. 58, 1419-1427.

108. Marbaix, G. and Gurdon, J.B. (1972) Biochim. Biophys. Acta. 281, 86-92.

109. Marbaix, G., Huez, G., Burny, A., Cleuter, Y., Hubert, E., LeClercq, M., Chantrenne, H., Soreq, H., Nudel, U. and Littauer, U.Z. (1975) Proc. Natl. Acad. Sci. USA 72, 3065-3067.

110. Marbaix, G. and Lane, C.D. (1972) J. Mol. Biol. 67, 517-524.

111. May, B.K. and Glenn, A.R. (1974) Aust. J. Biol. Sci. 27, 585-589.

112. McKeehan, W.L. (1974) J. Biol. Chem. 249, 6517-6526.

113. Merkel, C.G., Kwan, S.-P., and Lingrel, J.B. (1976) J. Biol. Chem. 251, 5512-5515.

113a. Moar, V.A., Gurdon, J.B., Lane, C.D. and Marbaix, G. (1971) J. Mol. Biol. 61, 93-104.

114. Moorman, A.F.M., Verkley, F.N., Asselbergs, F.A.M. and Grivell, L.A. (1977) in: Genetics and biogenesis of mitochondria (E. Kaudewitz, R.J. Schweyen, W. Bandlow and K. Wolf, eds) DeGruyter, Berlin pp385-399.

115. Moser, J.G., Jantzen, H., Schmidt, O., Wittman, R. and Krueger, G. (1975) Verh. Dtsch. Zool. Ges. 1974, 90-92.

116. Mous J., Peeters, B. and Rombauts, W. (1976) Mol. Biol. Rep. 2, 407-412.

117. Mous, J., Peeters, B. and Rombauts, W. (1977) Biochem. Biophys. Res. Commun. 72, 1111-1116.

118. Mous, J., Peeters, B., Van Belleghem, H. and Rombauts, W. (1977) Arch. Int. Physiol. Biochem. 85, 422-423.

118a. Mous, J., Peeters, B., Van Belleghem, H. and Rombaute, W. (1979) Eur. J. Biochem. 94, 393-400.

119. Nickol, J.M., Lee, K.-L., Hollinger, T.G. and Kenney, F.T. (1976) Biochem. Biophys. Res. Commun. 72, 687-693.

120. Nokin, P., Burny, A., Huez, G. and Marbaix, G. (1976) Eur. J. Biochem. 68, 431-436.

121. Nokin, P. Huez, G., Marbaix, G., Burny, A., and Chantrenne, H. (1976) Eur. J. Biochem. 509-517.

121a. Nudel, U., Soreq, H., Littauer, U.Z., Marbaix, G., Huez, G., LeClercq, M., Hubert, E. and Chantrenne, H. (1976) Eur. J. Biochem. 64, 115-121.

122. Nusse, R., Asselbergs, F.A.M., Salden, M.H.L., Michalides, R.J.A.M. and Bloemendal, H. (1978) Virology, 91, 106-115.

123. Ostertag, W., Gaedicke, G., Kluge, N., Melderis, H., Weimann, B. and Dube, S.K. (1973) FEBS Lett. 32, 218-222.

124. Ostro, M.J., Giacomo, D., Lavelle, D., Paxton, W. and Dray, S. (1978) Nature 274, 921-923.

125. Palmiter, R.D. (1974) J. Biol. Chem. 249, 6779-6787.

126. Palmiter, R.D. and Schimke, R.T. (1973) J. Biol. Chem. 248, 1502-1512.

127. Perry, R.P. and Kelley, D.E. (1973) J. Mol. Biol. 79, 681-696.

128. Phillips, J.A., Snyder, P.G. and Kazazian, H.H. (1977) Nature 269, 442-445.

129. Prensky, W. (1976) Meth. Cell Biol. 13, 121-152.

130. Princen, H.R.G., van Eekelen C.A.G., Asselbergs, F.A.M. and van Venrooij, W.J. (1979) Mol. Biol. Rep., in press.

131. Raj N.B.K. and Pitha, P. (1977) Proc. Natl. Acad. Sci USA 77, 1483-1487.

132. Rapoport, T.A., Thiele, B.J., Prehn, S., Marbaix, G., Cleuter, Y., Hubert, E. and Huez, G. (1978) Eur. J. Biochem. 87, 229-233.

133. Revel, M. and Groner, Y. (1978) Ann Rev. Biochem. 47, 1079-1126.

134. Reynolds, F.H., Premkumar, E. and Pitha, P.M. (1975) Proc. Natl. Acad. Sci. USA 72, 4881-4885.

135. Reynolds, R.K., van de Ven, W.J.M. and Stephenson, J.R. (1978) J. Virol 16, 1520-1535.

136. Rollins, J.W. and Flickinger, R.A. (1972) Science 178, 1204-1205.

137. Rosbash, J. and Ford, P.J. (1974) J. Mol. Biol. 85, 87-101.

138. Ross, J. and Knecht, D.A. (1978) J. Mol. Biol. 119, 1-20.

139. Ruderman, J.V. and Pardue, M.L. (1978) J. Biol. Chem. 253, 2018-2025.

139a. Rungger, D. and Tuerler, H. (1978) Proc. Natl. Acad. Sci. USA 75, 6073-6077.

140. Rutgers, T., Neeleman, L, van Vloten-Doting, L., Cleuter, Y., Hubert, E., Huez, G. and Marbaix, G. (1976) Arch. Int. Physiol. Biochem. , 654-655.

141. Rutgers, A.S. (1977) Thesis, State University of Leiden, Leiden, The Netherlands.

142. Salden, M., Asselbergs, F.A.M. and Bloemendal, H. (1976a) Nature 259, 696-699. (section V-2).

143. Salden, M.H.L., Selten-Versteeghen, A.-M. and Bloemendal, H. (1976b) Biochem. Biophys. Res. Commun. 72, 610-618.

144. Schaeffer, J.R., Trostle, P.K. and Evans, R.F. (1969) J. Biol. Chem. 244, 4284-4291.

145. Schroeder, J., Kreuzaler, F. and Schmock, J. (1977) FEBS Lett 81, 10-12.

146. Schwinghamer, M.W. and Symons, R.H. (1977) Virology 79, 88-108.

147. Seghal, P., Dobberstein, B. and Tamm, I. (1977) Proc. Natl. Acad. Sci. USA 74, 3409-3413.

148. Seghal, P.B., Soreq, H. and Tamm, I. (1978) Proc. Natl. Acad. Sci USA 75, 1614-1618.

149. Semancik, J.S., Conejero, V. and Gerhardt, J. (1977) Virology 80, 218-221.

150. Shatkin, A.J. (1976) Cell 9, 645-653.

151. Shimotohno, K., Kodama, Y., Hashimoto, J. and Miura, K.I. (1977) Proc. Natl. Acad. Sci. USA 74, 2734-2738.

152. Sippel, A.E., Stavrianopoulos, J.G., Schutz, G. and Feigelson, P. (1974) Proc. Natl. Acad. Sci. USA 71, 4635-4639.

153. Smith, M., Stavezner, J., Huang, R.C., Gurdon, J.B. and Lane, C.D. (1973) J. Mol. Biol. 80, 553-557.

154. Soreq, H., Nudel, U., Salomon, R., Revel, M. and Littaner, U.Z. (1974) J. Mol. Biol. 88, 233-245.

155. Spirin, A.S. (1969) Eur. J. Biochem. 10, 20-35.

156. Stacey, D.W. and Allfrey, V.G. (1976) Cell 9, 725-732.

157. Stevens, R.H., Askonas, B.A. and Welstead, J.L. (1975) Eur. J. Immunol. 5, 47-53.

158. Stevens, R.H. and Williamson, A.R. (1972) Nature 239, 143-146.

159. Stevens, R.H. and Williamson, A.R. (1973a) J. Mol. Biol. 78, 505-516.

160. Stevens, R.H. and Williamson, A.R. (1973b) J. Mol. Biol. 78, 517-525.

161. Stevens, R.H. and Williamson, A.R. (1973c) Nature New Biology 245, 101-104.

162. Stevens, R.H. and Williamson, A.R. (1975) Proc. Natl. Acad. Sci. USA 72, 4679.

163. Stoltzfus, C.M., Shatkin, A.J. and Banerjee, A.K. (1974) J. Biol. Chem. 248, 7993-

164. Tuohimaa, P. and Soederling, E. (1976) Acta Endocrinol 81, 593-597.

165. van der Donk, J.A.W.M. (1975) Nature 256, 674-675.

166. van der Donk, J.A., van Dam J.H. and Bazin, H. (1978) Nature 271, 479-481.

167. van der Donk, J.A., van Dam, R.H., Goudswaard, J., Hackeng, W. and Lips, C. (1976) Lancet ii, 1133.

168. van der Saag, P.T.M. (1975) Thesis, State University of Utrecht, Utrecht, The Netherlands.

169. van der Saag, P.T., Vlak, J.M., en de Greef, Th.F.J. (1976) Cell Diff. 4, 385-397.

170. van Vloten-Doting, L., Bol. J., Neeleman, L., Rutgers, T., van Dalen, D., Castel, A., Bosch, L., Marbaix, G., Huez, G., Hubert, E and Cleuter, Y., 1977 in: Nucleic Acid and protein synthesis in plants (L. Borograd and J.H. Weil, eds) Plenum. Publ. Col, New York, pp387-411.

171. van Zaane, D., Gielkens, A.J.L., Hesselink, W.G. and Bloemers,H.P.J. (1977) Proc. Natl. Acad. Sci. USA 74, 1855-1859.

172. Vassart, G., Brocas, H., Lecocg, R. and Dumont, J.E. (1975a) Eur. J. Biochem. 55, 15-22.

173. Vassart, G., Brocas, H., Nokin, P. and Dumont, J.E. (1973) Biochim. Biophys. Acta. 324, 575-580.

174. Vassart, G., Lecocq, G., Brocas, H. and Dumont, J.E. (1974) Biochim. Biophys. Acta 353, 261-265.

175. Vassart, G., Refetoff, S., Brocas, H., Dinsart, C. and Dumont, J.E. (1975b) Proc. Natl. Acad. Sci. USA 72, 3839-3843.

176. White, J.O., Hall, C., Lim, L. and Davison, A. (1975) Biochem. Soc. Trans. 3, 94-95.

177. Wiggins, T.L., Tucciarone, L.M. and Lanclos, K.D. (1978) Mol. Cell Biochem. 21, 145-151.

178. Williamson, R., Crosley, J. and Humphries, S. (1974) Biochemistry 13, 703-707.

179. Williamson, R., Drewienkiewicz, C.E. and Paul, J. (1973) Nature New Biol. 241, 66-68.

180. Williamson, R., Crossley, J. and Humphries, S. (1974) Dev. Biol. 40, 90-101.

180a. Woodland, H.R. (1974) Dev. Biol. 40, 90-101.

180b. Woodland, H.R. and Ayers, S.E. (1974) Biochem. J. 144, 11-19.

181. Woodland, H.R., Gurdon, J.B. and Lingrel, J.B. (1974) Dev. Biol. 39, 134-140.

182. Yang, S.-F. and Niu, M.C. (1977) Proc. Natl. Acad. Sci. USA 74, 1894-1898.

183. Yip, C.C., Hew, Ch-L, and Hsu, H. (1975) Proc. Natl. Acad. Sci. USA 72, 4777-4779.

184. Zehavi-Willner, T. and Lane C.D. (1977) Cell 11, 683-693.

Eur J Biochem 87 517–524 (1978)



# Synthesis of Lens Crystallins in *Xenopus* Oocytes as Determined by Quantitative Immunoprecipitation

Fred A M ASSELBERGS Walther J VAN VENROOIJ and Hans BLOEMENDAL

Department of Biochemistry University of Nijmegen

(Received December 19 1977)

Total poly(A)-containing calf lens mRNA was microinjected into *Xenopus* oocytes and synthesis of α, β, and γ-crystallins was demonstrated By a method of quantitative immunoprecipitation the rate of translation of purified 14 S $\alpha A_2$-crystallin mRNA was compared with translation of 9-S rabbit globin mRNA Maximal response of oocytes was obtained with virtually the same molar amounts of mRNA, taking into account the larger size of the $\alpha A_2$-crystallin mRNA Kinetics of translation were also very similar and both mRNAs were translated with similar rate and efficiency for at least two days It was estimated that 20 30 polypeptide chains per hour per mRNA molecule were synthesized

Microinjection of heterologous mRNA into *Xenopus* oocytes followed by the identification of the translation products has been achieved now for many kinds of mRNA, including the 14-S $\alpha A_2$-crystallin mRNA from calf lenses [1] In fact, the translational machinery of the oocyte seems to accept any cellular or viral mRNA from eukaryotes Only with a bacteriophage mRNA [2] and a mitochondrial mRNA [3] have no translation products been detected as yet The translational ability of oocytes renders these cells a very suitable model system to study how a cytoplasmic mRNA is translated in a eukaryotic cell However, such studies require quantitative data on the translation of purified mRNAs and these are only available in a few cases, notably for globin mRNAs from rabbit [4–7], mouse [6, 8], duck [9] and man [10] and recently for chicken ovalbumin mRNA [11] and encephalomyocarditis viral RNA [7] In all these cases the translation product was separated from the huge amount of endogenous proteins by chromatography on Sephadex beads or by gel electrophoresis In the present study we introduce a quantitative immunoprecipitation method as a possible technique to measure the rate of translation in oocytes of several mRNAs This can be done in spite of the high background activity in these cells We have used this method to compare in a quantitative way the translation characteristics of purified 14-S calf $\alpha A_2$-crystallin mRNA with those of rabbit globin mRNA

## EXPERIMENTAL PROCEDURE

Centrifugation gravity values are quoted as the average *g* values generated at particular speeds of rotation All buffers were standardized to the desired pH with concentrated HCl at 4 C and unless otherwise stated, all operations were carried out at 0–4 °C

### *Isolation of Lens mRNAs*

Calf lens polyribosomes, isolated as previously described [12, 13], were dissolved at 4 mg/ml in water and stored at − 80 C For isolation of lens mRNA the polyribosomes were extracted with phenol/chloroform according to Perry et al [14] RNA was precipitated by adding 0 1 vol of 2 M sodium acetate, pH 5 2, and 2 5 vol of ethanol and stored for 20 h at − 20 C The precipitated RNA was pelleted by centrifugation and dissolved in buffer A (application buffer 10 mM Tris/HCl, pH 7 0, containing 0 5 M NaCl, 1 mM EDTA and 0 5% sodium dodecylsulphate) and put on an oligo(dT)-cellulose column (Collaborative Research, $T_2$ grade) at a concentration of 3 mg/ml Chromatography was performed as described by Aviv and Leder [15] The poly(A)-containing fraction eluted with the low-salt buffer was precipitated with ethanol and stored at − 20 °C To remove the contaminating 18-S ribosomal RNA the procedure of Bantle et al [16] was followed with the modification that formamide was used instead of dimethyl-

sulfoxide No 18-S ribosomal RNA could then be detected in the fraction eluted with low salt buffer For translation purposes the purified RNA was dissolved in water at a concentration of 0 2 mg ml In some cases lens mRNAs were separated into 10 S and 14 S fractions by means of repeated sucrose gradient centrifugations [17] 95°, of the translation product of 14-S mRNA in a reticulocyte lysate comigrated with marker calf $\alpha A_2$-crystallin

*Isolation of Globin mRNA*

Total poly(A) containing RNA was isolated from reticulocyte polyribosomes as described above for calf lens polyribosomes and loaded onto an isokinetic sucrose gradient (15–35% w w) and centrifuged for 22 h in an SB283 rotor of an IEC ultracentrifuge The globin-mRNA containing fractions (9 S) were pooled precipitated with sodium acetate and ethanol and stored at – 20 C Before use the mRNA was dissolved in water at a concentration of 0 2 mg ml

*Microinjection of* Xenopus *Oocytes*

Ovaries were taken from *Xenopus* females, which were reared in the laboratory and which had not been injected with hormone [6] Oocytes of stage VI [18] were dissected and incubated in modified Barth s medium with follicle cells still adhering [2] Oocytes were injected individually using a Brinkmann MP-IV micromanipulator Micropipettes of 10–15 µM diameter were made from 9-µl Drummond microcaps in a glass microelectrode puller (model PN 3W Narishige Scientific Inst Labs Tokyo, Japan) and were attached to a 10-µl syringe (Unimetrix) filled with water A drop of petroleum ether (80–110 C) was used to seal off the mRNA solution With 1-nl accuracy up to 70 nl could be injected without damaging the oocytes Except when otherwise indicated 25 nl mRNA (0 2 mg ml) were injected Oocytes were labeled in Barth s medium with either 0 5 mCi ml L-[$^{35}$S]methionine (spec act 765 Ci mol) or L [2,5-$^{3}$H]histidine (spec act 47 Ci mmol) or L [4 5-$^{3}$H]-leucine (spec act 55 Ci mmol), all products from the Radiochemical Centre Amersham England After incubation at 19 C for various periods of time, ab normal-looking oocytes were discarded and oocytes were stored at – 20 C awaiting further processing

Thawed samples of five oocytes were homogenized in 0 5 ml buffer D (10 mM Tris/HCl pH 9 0 containing 50 mM NaCl) The homogenate was centrifuged at 5000 × *g* for 10 min and the pellet was reextracted with another 0 2 ml of buffer D The combined supernatants were extracted twice with diethylether to remove oocyte fat and were then lyophilized Oocyte proteins were taken up in 0 05 ml 10% glycerol containing 2% sodium dodecylsulphate 10% 2-mercaptoethanol and 0 01% bromophenol blue and analyzed by dodecylsulphate polyacrylamide gel electrophoresis

To measure protein synthesis a sample of oocyte homogenate was delivered into 0 5 ml 0 1 M NaOH After incubation for 30 min at 37 C protein was precipitated with trichloroacetic acid and counted on a filter Radioactivity not present in protein was determined by measuring total radioactivity in Aquasol (New England Nuclear) and subtracting acid precipitable radioactivity after correction for reduced efficiency in counting precipitated samples

*Immunoprecipitation of Lens Proteins*

Batches of five oocytes were homogenized in 0 5 ml NaCl P, dodecylsulphate (10 mM sodium phosphate buffer, pH 7 2, containing 0 9% NaCl, 0 3% Triton X-100, 0 5% sodium deoxycholate and 0 1% sodium dodecylsulphate) The homogenate was centrifuged at low speed and the pellet reextracted with 0 5 ml NaCl P$_i$/dodecylsulphate The combined supernatants were then centrifuged for 15 min in a Spinco Ti50 rotor at 200000 × *g* The pellet containing pigmented material was discarded About 90% of the radioactive protein was found in the supernatant To 0 7 ml of this supernatant, sufficient carrier antigen and antiserum were added (see legend to Table 1) together with 0 25 vol five times-concentrated NaCl/P$_i$/dodecylsulphate to correct for salt conditions The mixture was stored overnight at 4 C The precipitate was pelleted by centrifugation and processed for dodecylsulphate gel electrophoresis as described by Van Zaane et al [19] In some cases the immunoprecipitates were dissolved in 0 1 M NaOH in order to determine the amount of precipitated protein [20]

Antisera were prepared as described by Van Kamp et al [21] The antiserum raised against total α-crystallin reacted only with the acidic αA-subunit as judged by Ouchterlony immunodiffusion analysis This antiserum will be referred to as $\alpha A_2$-crystallin antiserum

*Dodecylsulphate Polyacrylamide Electrophoresis*

Oocyte extracts or immunoprecipitates were analyzed on 13% polyacrylamide gels according to Laemmli [22] with the modification that a gel slab was used instead of rods Staining and destaining were performed as described by Weber and Osborn [23] For detection of radioactive proteins gels were treated according to Bonner and Laskey [24] and processed for autoradiography as described previously [13]

*Assay of Globin Synthesis in Oocytes*

Oocytes were analyzed by dodecylsulphate gel electrophoresis as described above. After autoradiography the globin band was cut out of the gel slab, solubilized with a mixture of ammonia/hydrogen peroxide (1/19) overnight at 37 °C, and counted using Aquasol (New England Nuclear) as a scintillation fluid. Because we did not want to solubilize the enormous amount of unlabeled yolk proteins present in oocytes, the oocytes were homogenized without detergents. Under these conditions a varying amount of radioactive proteins remained entrapped in subcellular structures of oocytes. It was assumed that the amount of globin which was solubilized was always proportional to the total amount of radioactive protein which was solubilized. The correctness of this assumption was verified in some cases by running the insoluble part on a very large acrylamide gel.

## RESULTS

*Identification of the Translation Products by Gel Electrophoresis*

When oocytes were injected with total poly(A)-containing lens mRNA, several newly synthesized products appeared (Fig. 1 A – E) with mobilities similar to those of calf lens polypeptides (Fig. 1 F – I). $\alpha A_2$-crystallin could be identified especially easily, because it is the fastest-moving lens polypeptide. Other translation products comigrated with the $\beta$ and $\gamma$-crystallin fractions. The 14-S mRNA fraction produced only $\alpha A_2$-crystallin (Fig. 1 D), as was demonstrated previously [1], and this polypeptide is not coded for by the 10-S mRNA fraction (Fig. 1 C). This mRNA fraction codes primarily for $\alpha B_2$ and $\gamma$-crystallin (which comigrate in this electrophoresis system) and also for some $\beta$-crystallin polypeptides. Translation of 9-S globin mRNA is underestimated in comparison to the crystallins since the oocytes were labeled with [$^{35}$S]methionine and the globin molecule contains only one methionine residue.

*Immunoprecipitation of Calf Lens Proteins from Oocyte Lysates*

Immunoprecipitation can be used to identify and to quantify proteins simultaneously. In order to determine the conditions for quantitative immunoprecipitation of lens proteins from oocyte lysates, a constant amount of antiserum was added to the homogenate of oocytes that had been injected with total lens mRNA. Then increasing amounts of purified lens proteins were added. Maximal precipitation of radioactive lens protein was obtained when the carrier lens protein was present in half-saturating amounts with respect to the amount of antiserum. This is shown in

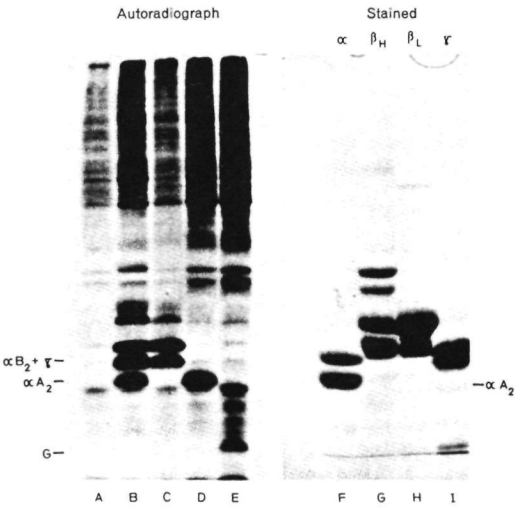



Fig. 1. *Electrophoretic analysis of microinjected oocytes.* After the injection of the mRNA (see Experimental Procedure) oocytes were labeled with [$^{35}$S]methionine for 16 h and analyzed on a slab gel. For the purpose of identification the positions of crystallin polypeptides and globin (G) in the gel are indicated. Oocytes were injected with (A) 25 nl water, (B) total lens mRNA (25 nl, 0.2 mg/ml), (C) 10-S lens mRNA (25 nl, 0.2 mg/ml), (D) 14-S lens mRNA (25 nl, 0.2 mg/ml) and (E) 9-S rabbit globin mRNA (25 nl, 0.2 mg/ml). A gel with α-crystallin (F), two classes of $\beta$-crystallin (G, H) and $\gamma$-crystallin (I) was stained with Coomassie brilliant blue

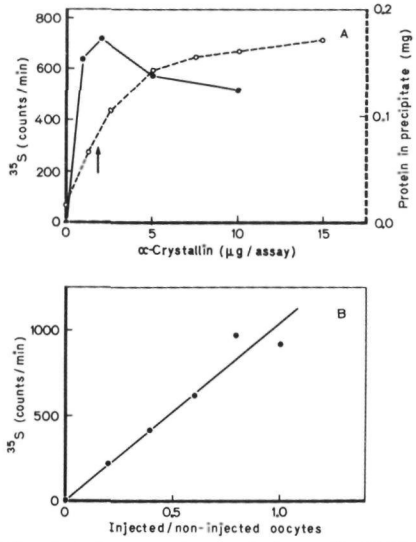



Fig. 2. *Quantitative immunoprecipitation of $\alpha A_2$-crystallin.* (A) 0.5 ml of lysate of oocytes, which had been injected with total lens mRNA and incubated in Barth's medium with [$^{35}$S]methionine and homogenized as described in Experimental Procedure was mixed with 0.05 ml rabbit antiserum raised against α-crystallin. Different amounts of carrier α-crystallin were added. Radioactivity (●——●) and protein content (○——○) of immunoprecipitates were determined as described in Methods. The arrow indicates the amount chosen for quantitative immunoprecipitation in (B). Linearity of immunoprecipitation was tested by mixing, in different ratios, a lysate of oocytes, injected with lens mRNA, with a lysate of non-injected oocytes

Fig. 2A for $\alpha A_2$-crystallin, but similar results were obtained for the β and γ-crystallin polypeptides (data not shown). When lysates of oocytes injected with lens mRNA were mixed with lysates of non-injected oocytes in different ratios, the radioactivity present in the immunoprecipitates was directly proportional to the mixing ratio. This was found not only with antiserum against $\alpha A_2$-crystallin (Fig. 2B), but also with the translation products of 10-S mRNA, namely β-crystallin (data not shown) and γ-crystallin (Fig. 3E–J). A second immunoprecipitation, performed on the supernatant from the first immunoprecipitation, always contained less than 5% of the radio-

Table 1. *Efficiency of immunoprecipitation*
Oocytes were injected with lens mRNA and labeled with [$^{35}$S]-methionine for 20 h. Oocytes were then homogenized and processed for immunoprecipitation as described in Methods. Conditions for quantitative immunoprecipitation were determined as in the legend to Fig. 2. 0.7 ml oocyte lysate was immunoprecipitated after addition of 2 μg α-crystallin and 0.1 ml antiserum or 4 μg β-crystallin and 0.35 ml antiserum or 4 μg γ-crystallin and 0.1 ml antiserum

| Antiserum directed against | $^{35}$S-labeled protein/oocyte | | |
|---|---|---|---|
| | input | first immuno-precipitation | second immuno-precipitation |
| | counts/min | | |
| $\alpha A_2$-crystallin | 57740 | 3600 | 120 |
| β-Crystallin | 57740 | 2750 | 110 |
| γ-Crystallin | 57740 | 5000 | 175 |

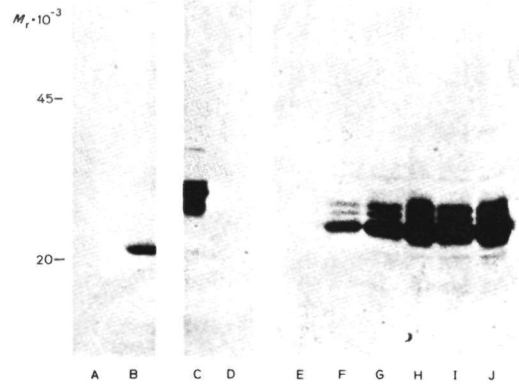



Fig. 3. *Electrophoretic analysis of immunoprecipitates.* Oocyte-derived lens proteins were analyzed by quantitative immunoprecipitation with antiserum specific for $\alpha A_2$-crystallin (A, B), β-crystallin (C, D) and γ-crystallin (E–J). Immunoprecipitates of oocytes injected with water (A, D, E) or lens mRNA (B, C, F–J) are shown. Immunoprecipitation of γ-crystallin was performed with mixtures in different ratios of lysates of oocytes injected with water or lens mRNA (E–J). The ratios are the same as used in Fig. 2B for $\alpha A_2$-crystallin antiserum. The amount of immunoprecipitated material was directly proportional to the mixing ratio. Conditions of immunoprecipitation are as described in Table 1

activity of the first one (Table 1) indicating that more than 95% of the translation product is precipitated in a one-step procedure and thus that the immunoprecipitation is quantitative It was also possible to use the supernatant of the first immunoprecipition for the detection and quantification of a second protein

Under these conditions of quantitative immunoprecipitation only $\alpha A_2$-crystallin was precipitated with the $\alpha A_2$-crystallin antiserum (Fig 3B) demonstrating the specificity of the precipitation reaction In the immunoprecipitate with antiserum against the $\beta$-crystallin fraction (Fig 3C) a number of bands were seen, all with typical $\beta$-crystallin mobility [25] The faint fast-moving band probably represents $\alpha A_2$-crystallin because the antiserum used showed a trace of activity against this protein Immunoprecipitation of $\gamma$ crystallin revealed the presence of one major and two minor bands (Fig 3E–J)

### *Dose-Response Relationship of Translation of Exogenous mRNA in* Xenopus *Oocytes*

One of the most characteristic features of a translation system is the amount of mRNA needed to obtain maximal response Increasing amounts of purified 14-S $\alpha A_2$-crystallin mRNA were therefore injected and the amount of $\alpha A_2$-crystallin synthesized was measured by quantitative immunoprecipitation (Fig 4B) Maximal $\alpha A_2$-crystallin synthesis was obtained at about 11–13 ng mRNA oocyte, while total oocyte protein synthesis remained more or less constant (Fig 4A) A similar dose-response curve could be obtained for 9-S globin mRNA (Fig 4C) In this case 7–8 ng was needed for maximal globin synthesis The absolute amounts of translation products are not comparable, since oocytes were labeled with [$^{35}$S]-methionine in the case of $\alpha A_2$-crystallin mRNA and with [$^3$H]histidine when globin mRNA was injected

### *Kinetics of mRNA Translation in Oocytes*

mRNA microinjected into oocytes is not translated immediately at maximal rate as is the case in most cell free systems In an oocyte, translation activity of the injected mRNA rises for some hours until a plateau of maximal rate of translation is reached The rate of translation may then remain constant for periods up to two weeks in the case of stable mRNAs [6] In our experiments injected oocytes were labeled for 4-h periods at various times after injection of the mRNAs (Fig 5) Both $\alpha A_2$-crystallin mRNA and globin mRNA were already translated at maximal rate during the labeling period between 4 and 8 h after injection They were completely stable for at least two days In another experiment longer incubation times up to four days were used with essentially the same results

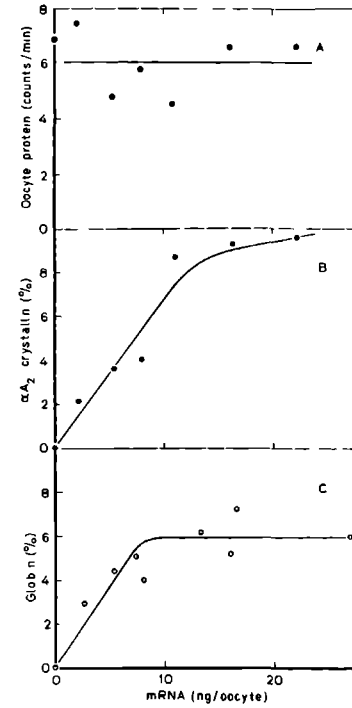



Fig 4 *Dose response relationship of mRNA translation* Oocytes were injected with different amounts of 14 S lens mRNA (A B) or 9 S globin mRNA (C) and labeled for 18 h with [$^{35}$S]methionine or [$^3$H]histidine respectively Total oocyte protein synthesis (A) was measured as described in Methods The amount of $\alpha A_2$ crystallin synthesized was measured by quantitative immunoprecipitation Globin synthesis was assayed by determination of the radioactivity in the globin band of a polyacrylamide gel The amount of specific polypeptides synthesized is expressed as a percentage of total acid precipitable radioactivity In control oocytes that had not been injected with globin mRNA a small amount (0 5%) of radioactivity migrated in the globin region of the gel This background has been corrected for in (C)

### *Translational Efficiency of 14-S $\alpha A_2$-Crystallin mRNA*

Translational efficiency can be estimated in oocytes, provided that the specific activity of the intracellular amino acid pool is known In our case the specific activity of leucine and histidine pools was estimated using the total amino acid content of stage V oocytes cultured in amino-acid-free medium, which very likely is identical to the kinetic pool [9] Such a procedure has also been applied in the case of globin mRNA [2] and ovalbumin mRNA [11] Data on translational efficiency of 14-S mRNA are given in Table 2 and compared with similar data on globin mRNA The translational efficiency of both mRNAs was very similar, although the estimates varied considerably

between experiments In a second type of experiment both mRNAs were simultaneously injected into the same cells (Table 2, expt C), which were then labeled with leucine In such experiments translational efficiencies can be compared under identical conditions In view of the saturation effect obtained in other experiments it is important to note that in experiments A and C both mRNAs were tested in a concentration range where the amount of translation product correlated linearly with the amount of mRNA injected (Fig 4) The results showed that both mRNAs were translated with roughly equal efficiencies

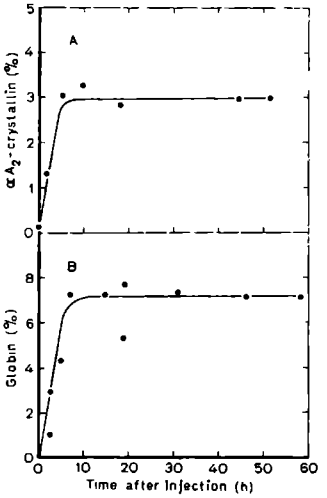



Fig 5 *Kinetics of translation of mRNA in* Xenopus *oocytes* 5 ng 14 S lens mRNA was injected into oocytes (A) Batches of 10 oocytes were labeled for 4-h periods with [$^{35}$S]methionine at different times after injection The middle of the labeling period has been indicated in the figure Similarly oocytes injected with 5 ng globin mRNA were labeled with [$^{3}$H]histidine (B) Translation was measured as described in the legend of Fig 4

## DISCUSSION

When total poly(A)-containing lens mRNA was injected into *Xenopus* oocytes a number of lens proteins was synthesized (Fig 1) These proteins could be identified by a combination of immunoprecipitation with antisera against α, β, and γ-crystallins and gel electrophoresis With antiserum raised against total α-crystallin only the $\alpha A_2$-subunit was precipitated (Fig 3B) Other newly synthesized polypeptides could be identified as β-crystallins or γ-crystallin (Fig 3) The 14-S fraction of lens mRNA produced $\alpha A_2$-crystallin exclusively and this polypeptide, as expected, was absent after translation of 10-S mRNA (Fig 1) This confirms previous results obtained by Berns et al [1]

In order to measure crystallin synthesis in a quantitative way, a method was developed for direct immunoprecipitation of different crystallin classes from oocyte lysates This method involved homogenization of the oocytes with the aid of detergents, since it appeared to be impossible otherwise to solubilize the translation products completely In our procedure aspecific

Table 2 *Efficiency of translation of mRNAs in* Xenopus *oocytes*

The amounts of mRNA are given assuming no leakage or breakdown of mRNA In a separate experiment, iodinated mRNA was injected and 98 ± 6% of the radioactivity could be recovered from the oocytes after a 6 h incubation period (Asselbergs unpublished results) The amounts given on a molar basis assume a molecular weight of 510000 for 14 S $\alpha A_2$ crystallin mRNA [27] and of 200000 for globin mRNA Results given within brackets were obtained from a duplicate experiment with oocytes from the same *Xenopus* The average amino acid content of oocytes was measured from a perchloric acid extract from batches of 1000 oocytes from three different frogs after 12 h culture *in vitro* Similar values for oocytes in culture were reported by Bravo et al [26] The specific activity is the total soluble radioactivity per oocyte calculated as described in Methods divided by the amino acid content per oocyte The amounts of translation products were calculated remembering that $\alpha A_2$ crystallin contains 14 leucine residues and β-globin contains 18 leucine and 9 histidine residues In calculating the amount of polypeptide it was noted that only β globin mRNA is translated efficiently in oocytes in the absence of hemin [5 6 9] (and our own observations) and it was assumed that β globin mRNA makes up 41%, of the polysomal mRNA of rabbit reticulocytes [30]

| Expt | Injected mRNA | mRNA oocyte | | Radioactivity per oocyte incorporated into translation products | Precursor amino acid | Amino acid content per oocyte | Specific activity | Translation product per oocyte | Polypeptides mRNA |
|---|---|---|---|---|---|---|---|---|---|
| | | weight basis | molar basis | | | | | | |
| | | pg | pmol | dis min$^{-1}$ h$^{-1}$ | | pmol | dis min$^{-1}$ pmol$^{-1}$ | pmol h | molecule$^{-1}$ h$^{-1}$ |
| A | 14 S | 1125 | 0 0022 | 2246 | leucine | 143 | 3393 | 0 047 | 21 5 |
| B | 9-S | 8100 | 0 0406 | 2700 | histidine | 203 | 1642 | 0 426 | 26 2 |
| C | 14 S | 562 | 0 0011 | 325 (400) | leucine | 143 | 1032 (1432) | 0 022 (0 020) | 20 5 (18 2) |
| | 9-S | 2500 | 0 0125 | 1363 (1676) | leucine | 143 | 1032 (1432) | 0 074 (0 065) | 14 7 (12 8) |

precipitation is virtually absent (Fig 3) therefore our procedure appears to represent an improvement when compared with the direct immunoprecipitation used by Berridge et al [32] At optimal concentrations of the antiserum and the carrier antigen the immunoprecipitation was shown to be linear with the input of radioactive crystallin (Fig 2B and 3I – J) and more than 95 of the radioactive translation product was precipitated It is therefore clear that this method can be used to quantify the amount of crystallins synthesized in oocytes In fact if the proper antisera are available a wide variety of translation products can be monitored in oocytes using this method

The translation of purified 14-S $\alpha A_2$-crystallin mRNA in oocytes was compared with 9 S globin mRNA On a weight basis about twice as much 14 S mRNA was needed to obtain maximal $\alpha A_2$-crystallin synthesis relative to the endogenous synthesis as was needed with 9 S mRNA however when the larger size of the 14 S messenger ($M_r$ 510000 cf 200000 for globin mRNA [27]) is taken into account about 30 less 14 S mRNA was necessary on a molar basis One of the possible explanations of this phenomenon might be that in our comparison the functional amount of globin mRNA was overestimated because for example we did not take into account that in the absence of hemin the α globin mRNA is hardly translated in oocytes (Table 2) Of course absolute translation levels would be dependent on oocyte size and probably other (physiological) conditions as well

Comparison of the kinetics of translation of both mRNAs showed that 14-S mRNA and 9-S mRNA were both stable for at least two days It has been found that a poly(A) track of at least 32 adenyl residues is necessary to stabilize globin mRNA in oocytes [28] 14 S $\alpha A_2$ crystallin mRNA contains a poly(A) stretch of about 50 – 100 adenyl residues [29] and therefore would be expected to be stable in oocytes

It was estimated that each hour about 20 – 30 polypeptide chains were coded for by an injected mRNA molecule (Table 2) It was concluded that the efficiency of translation of 14-S $\alpha A_2$-crystallin mRNA was at least equal or perhaps slightly better than globin mRNA translation Gurdon [30] found a rate of 20 $\beta$-globin molecules (molecule mRNA)$^{-1}$ h$^{-1}$ and the synthesis of 10 ovalbumin molecules (molecule mRNA)$^{-1}$ h$^{-1}$ was reported by Chan et al [11] who used the same estimation method of translational efficiency as used here From these data it seems that when mRNAs from different specialized eukaryotic cells are injected into *Xenopus* oocytes, all are translated with more or less the same efficiency Quantitative studies on mRNA translation in oocytes have been restricted to a very limited number of mRNAs partly because a general method of quantification of the translation products was not available The procedure of quantitative immunoprecipitation presented here might solve this problem

These investigations have partly been carried out under auspices of the Netherlands Foundation for Chemical Research (S O N) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z W O) We thank Miss M Koopmans for skilful technical assistance Dr I Cohen for a gift of 14 S lens mRNA and Prof F Katz for critical reading of the manuscript

## REFERENCES

1 Berns A J M van Kreijkamp M Bloemendal H & Lane C D (1972) *Proc Natl Acad Sci U S A 69* 1606 1609
2 Gurdon J B Lane C D Woodland H R & Marbaix G (1971) *Nature Lond 233* 177 182
3 Moorman A J M Verkley J N Asselbergs F A M & Grivell L A (1977) in *Genetics and Biogenesis of Mitochondria* (Kuroda K Tuppy H Schweyen R J Bandlow W & Wolf K eds) pp 385 399 De Gruyter Berlin
4 Moar V A Gurdon J B Lane C D & Marbaix G (1971) *J Mol Biol 61* 93 104
5 Righetti B Grimm A M Comi P Ottolenghi S & Rungger D (1973) *Nat New Biol 246* 99 – 102
6 Gurdon J B Lindsell J B & Marbaix G (1973) *J Mol Biol 80* 539 551
7 Jaskes R A Mills A D Gurdon J B & Partington G A (1977) *Cell 11* 345 352
8 Marbaix G & Gurdon J B (1972) *Biochim Biophys Acta 281* 86 93
9 Lane C D Gregory C M & Morel C (1973) *Eur J Biochem 34* 219 – 227
10 Manitius G M Ramirez F Cann A Marks P A & Bank A (1976) *J Clin Invest 58* 1419 1427
11 Chan L Kohler P O & O'Malley B (1976) *J Clin Invest 57* 576 585
12 Bloemendal H Schoenmakers J J G Zweers A Matze R & Benedetti E L (1966) *Biochim Biophys Acta 123* 217 – 220
13 Berns A J M & Bloemendal H (1974) *Methods Enzymol 30* 675 – 694
14 Perry R P LaTorre J Kelley D E & Greenberg J R (1972) *Biochim Biophys Acta 262* 220 226
15 Aviv A & Leder P (1972) *Proc Natl Acad Sci U S A 69* 1408 – 1412
16 Bantle J A Maxwell I H & Hahn W E (1976) *Anal Biochem 72* 413 427
17 Cohen L H Smits D P E M & Bloemendal H (1976) *Eur J Biochem 67* 563 573
18 Dumont J N (1972) *J Morphol 136* 153 – 180
19 Van Zaane D Gielkens A J L Dekker Michielsen M J A & Bloemers H P J (1975) *Virology 67* 544 552
20 Lowry O H Rosebrough N J Farr A L & Randall R J (1951) *J Biol Chem 193* 265 275
21 Van Kamp G J Smeets G W M & Hoenders H J (1973) *Biochim Biophys Acta 303* 194 197
22 Laemmli U K (1970) *Nature (Lond) 227* 680 – 685
23 Weber K & Osborn M (1969) *J Biol Chem 224* 4406 – 4412
24 Bonner W M & Laskey R A (1974) *Eur J Biochem 46* 83 88
25 Herbrink P van Westreenen H & Bloemendal H (1975) *Exp Cell Res 20* 541 – 548
26 Bravo R Salazar E & Allende J E (1976) *Exp Cell Res 103* 106 174

27 Berns, A , Janssen, P & Bloemendal, H (1974) *Biochem Biophys Res Commun 59*, 1157–1164
28 Nudel, U , Soreq H , Littauer U Z , Marbaix, G , Huez, G , Leclercq, M Hubert, E & Chantrenne, H (1976) *Eur J Biochem 64*, 115 121
29 Lavers, G C , Chen, J H & Spector, A (1974) *J Mol Biol 82*, 15–25
30 Gurdon, J B (1973) *Acta Endocrinol Suppl 180*, 225 - 243
31 Phillips, J A , Snyder, P G & Kazazian H H , Jr (1977) *Nature (Lond ) 269*, 442–445
32 Berridge, M V & Lane, C D (1976) *Cell, 8*, 283 – 297

F A M Asselbergs, W J Van Venrooij, and H Bloemendal, Laboratorium voor Biochemie, Katholieke Universiteit Nijmegen, Geert Grooteplein Noord 21, NL-6525 EZ Nijmegen, The Netherlands

Eur J Biochem 94 249 254 (1979)

III - 3

# Messenger RNA Competition in Living *Xenopus* Oocytes

Fred A M ASSELBERGS Walther J VAN VENROOIJ and Hans BLOEMENDAL

Biochemisch Laboratorium Katholieke Universiteit Nijmegen

(Received October 30 1978)

When calf lens crystallin mRNA and rabbit globin mRNA are competing for factors limiting protein synthesis in living *Xenopus* oocytes no mRNA species is preferentially selected for translation Differences in the intrinsic translational efficiency of the mRNA species exist but the relative efficiencies are the same at high and low mRNA concentrations mRNAs already being translated, in particular endogenous oocyte mRNAs, are less sensitive to competitive inhibition by injected mRNAs As injected mRNAs gradually become incorporated into the protein synthesizing machinery of the oocyte, they acquire the same status as the oocyte's own active mRNAs Exogenous mRNAs thus become endogenous mRNAs

These results, together with previous estimates of the translational efficiency of injected heterologous mRNA species, are compatible with the assumption that a large proportion of the endogenous mRNAs is not competing for the translational apparatus of the oocyte and, therefore, probably is present in a temporarily inactivated form

*Xenopus* oocytes can be microinjected with controlled amounts of mRNA and thus provide an excellent system to study how protein synthesis is regulated in an intact eukaryotic cell [1] Microinjected mRNAs, for example those coding for duck [2] or rabbit [3] hemoglobin polypeptides or for calf lens $\alpha A_2$ crystallin [4,5], are very efficiently translated in oocytes, but they do not increase the total amount of protein synthesized by the oocyte [2,3,5] Protein synthesis of *Xenopus* oocytes, therefore, is limited by some component of the translational apparatus and not by the mRNA [3] Unlike the situation in many eukaryotic cells, mRNA translation in oocytes is not regulated at the initiation step of protein synthesis (more accurately, the ribosome binding process), nor does the elongation of polypeptides limit protein synthesis in oocytes These conclusions can be deduced from the observation that an increase of the amount of microinjected globin mRNA did not lead to a shift in the size of the globin-synthesizing polyribosomes [6] The factor limiting and thus regulating protein synthesis in oocytes, therefore, either affects initiation and elongation of polypeptides simultaneously or influences the recruitment [3] of the mRNA for protein synthesis The pioneering work of Laskey and coworkers has already shown that this limiting factor is a normal component of the polyribosomes The aim of the present series of experiments was to verify whether or not the regulatory factor(s) display(s) a selectivity for certain mRNA species

## MATERIALS AND METHODS

A tritium-labeled mixture of 15 amino acids was purchased from the Radiochemical Centre, Amersham Microinjection of purified rabbit globin mRNA and calf lens mRNA into *Xenopus laevis* oocytes as well as the assay of their respective translation products, have been detailed previously [5] Amounts of mRNA injected (expressed as ng/oocyte) are not corrected for the sizes of globin mRNA (9 S) or lens mRNA (10–14 S) Translation products from batches of 10 oocytes were quantified and expressed as counts $min^{-1}$/oocyte

## RESULTS AND DISCUSSION

### *Time Course of Translation of Microinjected mRNAs*

When mRNAs are injected into *Xenopus* oocytes, maximal rates of translation are only reached after several hours [5,7,8] Since a very slow rate of establishment has been found for 30-S vitellogenin mRNA relative to that of 18-S serum albumin mRNA [8], it was suggested that the rate of establishment is a function of the size of the mRNA In our experiments maximal translation of the calf lens crystallin mRNAs occurred after about 7 h (Fig 1B) $\alpha A_2$-crystallin, the major 20000-$M_r$ lens polypeptide, is coded for by an exceptionally large mRNA (14 S) [9], as compared to 10-S mRNAs coding for other 20000-$M_r$ polypeptides, for example the $\beta$ and $\gamma$-crystallin subunits [10] How-

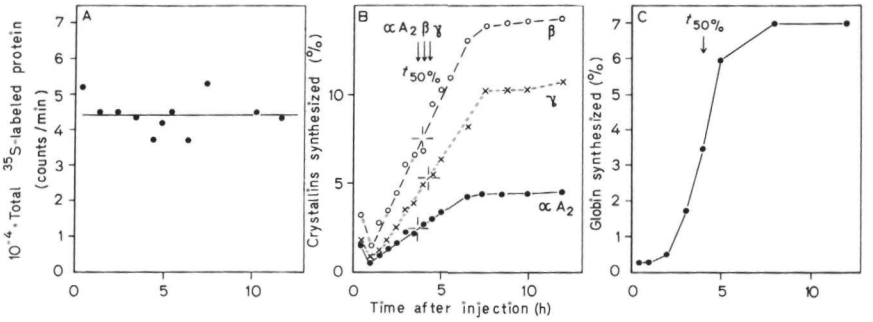



Fig. 1. *Rate of translation of microinjected mRNAs in* Xenopus laevis *oocytes.* Oocytes were labeled for 1-h periods with 0.5 mCi/ml L-[$^{35}$S]-methionine (A, B) or L-[$^{3}$H]histidine (C) at various times after injection of the mRNA. Calf lens and rabbit globin mRNAs were isolated as described earlier [5]. (A) Acid-precipitable radioactivity of oocytes injected with 5 ng lens mRNA. (B) Synthesis of the various types of lens crystallins was measured by quantitative immunoprecipitation [5] and expressed as percentage of the total radioactive protein. (C) Rabbit globin synthesis in oocytes injected with 5 ng 9-S mRNA was assayed by polyacrylamide gel electrophoresis [5] and is expressed as percentage of the total radioactive protein (about 54000 counts/min per oocyte). The time at which 50% of maximal translation was observed, $t_{50\%}$, is indicated. (Data of A and B were obtained with oocytes from a different frog than the data of C)

ever, translation of the large $\alpha A_2$-crystallin mRNA occurred with similar kinetics to the translation of the smaller lens mRNAs (Fig. 1 B). Rabbit globin mRNA (9 S), which in the absence of hemin was translated mainly into $\beta$-globin [11], reached its maximal rate of translation 6 h after injection (Fig. 1 C). The rate of establishment of all our mRNA species, therefore, is comparable to that reported for serum albumin mRNA [8]. While translation of the exogenous mRNAs increased with time, a concomitant decrease in the translation of the oocyte endogenous mRNAs was observed, resulting in a constant rate of total protein synthesis (Fig. 1 A). Apparently after about 6–7 h an equilibrium has been established between exogenous and endogenous protein synthesis.

*Simultaneous Injection of Two mRNA Species*

In another kind of experiment a constant amount of one mRNA species was injected together with a varying amount of a second mRNA species. Oocytes were labeled for 12 h starting 6 h after injection. In order to label all newly synthesized proteins to a comparable specific activity, a mixture of radioactive amino acids rather than a single amino acid was used [3]. The radioactivity present in different regions of a polyacrylamide gel, specified in Fig. 2, was directly measured in a liquid scintillation counter [5]. Interpretation of the results of this type of experiment focuses on three aspects, as follows.

*Exogenous mRNAs versus Endogenous mRNA.* As reported by others [3], synthesis of all endogenous proteins was equally affected by the injection of foreign mRNAs (Fig. 2). Therefore, the inhibition of the

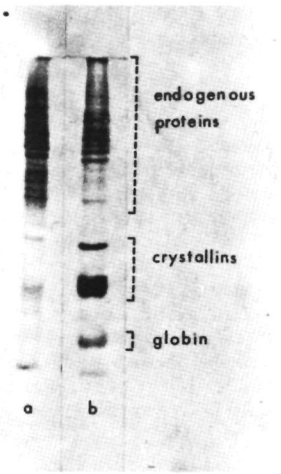



Fig. 2. *Translation of rabbit globin and calf lens mRNAs.* Oocytes were microinjected with 40 nl distilled water (a) or with 6 ng lens mRNA plus 5 ng globin mRNA (b). Oocytes were labeled for 12 h starting 6 h after injection with 0.5 mCi/ml $^{3}$H-labeled L-amino acids (Radiochemical Centre, Amersham), homogenized and subjected to dodecylsulphate/polyacrylamide gel electrophoresis as detailed previously [5]. Positions of 'representative' oocyte proteins (see text), lens crystallins and globin are indicated

synthesis of a group of oocyte polypeptides, which can be well separated from the translation products of globin and lens mRNA, is representative for that of all oocyte polypeptides (Fig. 3 A and E). Some batches of oocytes translate exogenous mRNAs much better

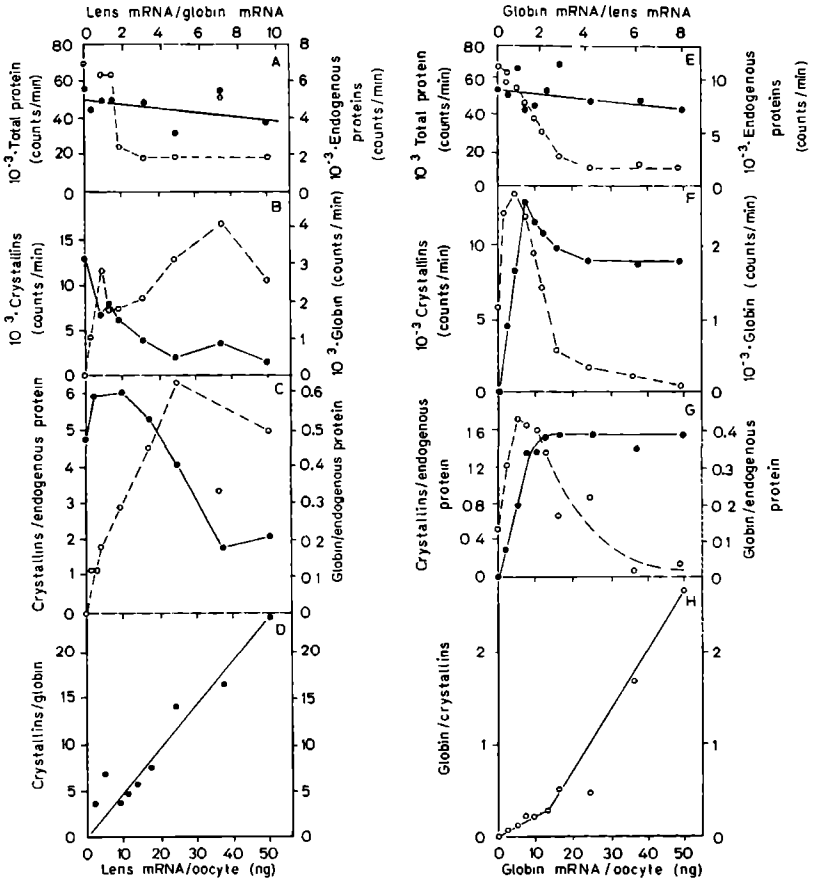



Fig 3 *Competition between globin, lens and endogenous mRNAs* Oocytes were microinjected with 5 ng rabbit globin mRNA plus increasing amounts of lens mRNA (A–D) or with 6 ng lens mRNA plus increasing amounts of globin mRNA (E–H) Labeling procedures were as described in the legend to Fig 2 Incorporation into total protein (A F ● ●) was measured as radioactivity precipitable with trichloroacetic acid The newly synthesized proteins were also subjected to dodecylsulphate/polyacrylamide gel electrophoresis as in Fig 2 and the radioactivity present in the indicated regions of the gel was measured as previously described [5] The proteins referred to as endogenous (A E ○ ○) are oocyte polypeptides with $M_r$ larger than 40000 Incorporation values for lens crystallins (B, F ○ - ○) and globin (B, F ● ●) have been corrected for comigrating endogenous proteins of the oocytes Crystallin synthesis (C, G ○ - ○) and globin synthesis (C, G ● ●) have also been expressed as a function of the synthesis of endogenous proteins with $M_r$ larger than 40000 The ratio crystallins globin synthesized (D H) is plotted against the ratio lens mRNA globin mRNA (indicated on top of the figure) The data shown in this figure and in Fig 4 were obtained with sibling oocytes

than other batches [1] Such oocytes are colloquially referred to as 'good' oocytes Fig 2 and 3 depict the results obtained with such 'good' oocytes For example, in Fig 3G the ratio globin/endogenous protein reaches a maximum of 0 4, whereas with oocytes obtained from a different frog, which were injected with the same mRNA mixtures, a maximal ratio of only 0 03 was reached ('bad' oocytes) Similarly values of 1.6 and 0 16 were obtained for the maximal ratio lens crystalline/endogenous proteins However, the general form of the competition curves of the two exogenous mRNAs (Fig 3C and G) was exactly the same in both 'good' and 'bad' oocytes The main difference between these oocytes, therefore, seems to be the extent of inhibition of the endogenous protein synthesis, which in 'good' oocytes may reach up to 70% (Fig 3A and E), but in 'bad' oocytes only 20–30% (not shown) In all batches of oocytes endogenous

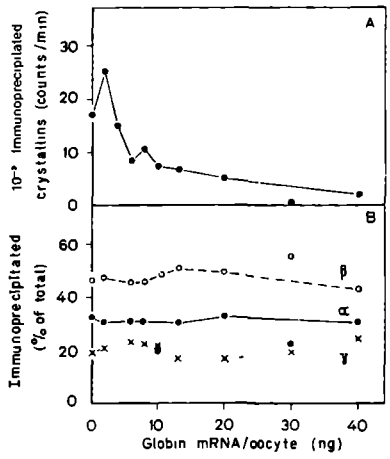



Fig 4 *Competitive inhibition of translation of different lens mRNAs by globin mRNA* Oocytes were injected with a constant amount of lens mRNA and increasing amounts of globin mRNA as in Fig 3 (*F–H*) Newly synthesized lens crystallins were quantified by immunoprecipitation with antisera to αA (● – ●) β (○– ○) and γ crystallin (×– ×) respectively [5] The sum of the three immunoprecipitates is shown in (A) whereas in (B) radioactivity in each of the immunoprecipitates is expressed as a percentage of the sum (The oocytes used in this experiment were obtained from the same frog as in Fig 3)

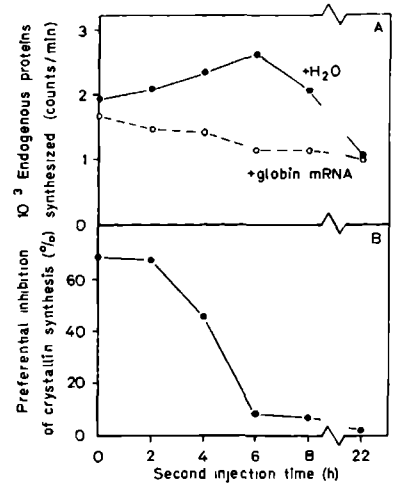



Fig 5 *Subsequent injection of two mRNAs into* Xenopus *oocytes* (A) Oocytes were injected at $t = 0$ with 25 nl calf lens mRNA (0 2 mg ml) and at the times indicated with either 25 nl distilled water (● ●) or rabbit globin mRNA (2 mg ml) (○ ○) Oocytes were labeled with $^3$H labeled t amino acids for 6 h starting from $t = 28$ h Translation products were subjected to dodecyl sulphate polyacrylamide gel electrophoresis and incorporation into a representative group of oocyte proteins (those with $M_r$ larger than 40000) is shown in the figure (B) The preferential inhibition of crystallin synthesis as a function of the time of the second injection (globin mRNA) The preferential inhibition is defined as the decrease in the ratio of the crystallin synthesis to the synthesis of the representative oocyte proteins caused by the presence of globin mRNA rather than just water in the second injection Therefore 0% inhibition in (B) implies an equal degree of inhibition of the lens crystallin and oocyte endogenous protein synthesis (The oocytes used in this experiment were obtained using a different frog than in Fig 3 and 4 and Table 1)

protein synthesis was inhibited by the translation of the foreign mRNA Our results thus confirm the absence of a large spare translational capacity in amphibian oocytes The concept of a spare capacity for mRNA translation was originally introduced by Gurdon's group [12], but rejected in a more recent paper [3]

*One Exogenous mRNA versus Another* The amount of crystallin produced per mRNA molecule was consistently several times higher than the amount of globin synthesized per mRNA molecule (cf the right and left-hand scales of Fig 3B and F) Both mRNA preparations contain apparently an equal content of functional molecules, as judged from mRNA saturation experiments in an mRNA dependent reticulocyte lysate [13] The difference may partly be caused by the reduced efficiency of the α-globin mRNA translation in oocytes, when injected without hemin [11] Only about 20% of the synthesized globin was found to be α-globin (data not shown), while the α-globin mRNA constitutes about 60% of the globin mRNA isolated from reticulocyte polyribosomes [14] But even if this is taken into account, a 2–3-fold difference in efficiency of translation between β globin mRNA and the mRNAs for the ($\alpha A_2$, β, γ) crystallins remains A similar difference has been reported previously by us, when the translation of β globin mRNA was compared with the translation of simultaneously injected 14-S $\alpha A_2$-crystallin mRNA [5] Co-injection of increasing amounts of lens mRNA with the globin mRNA decreased the synthesis of globin by the oocytes (Fig 3B) In the reverse experiment crystallin synthesis decreased when lens mRNA was injected together with increasing amounts of globin mRNA (Fig 3F) Such a competition of two exogenous mRNAs in oocytes has also been demonstrated by Gurdon's group [1, 3] We also found an almost linear correlation between the input ratio (globin mRNA/lens mRNA) and the output ratio (globin/crystallins) (Fig 3D and H) Apparently (β )globin mRNA and lens mRNA in oocytes compete for a factor which does not discriminate between these mRNA species Furthermore, the factor also does not discriminate between the different species of lens mRNA, since the ratios of $\alpha A_2$, β and γ-crystallin polypeptides, assayed by quantitative immunoprecipitation [5], re-

Table 1 *Subsequent injection of two mRNAs into* Xenopus *oocytes*
Oocytes were injected with mRNAs labeled and subjected to gel electrophoresis as indicated in Fig 5 (oocytes were taken from a different frog to the one used in Fig 5) From incorporation values for the crystallin and the globin regions of the gel incorporation due to the synthesis of oocyte endogenous proteins of similar size has been subtracted Values for endogenous proteins include those comigrating with globin and crystallin

| First injection | Second injection | Time of second injection | Globin | Crystallins | Endogenous proteins |
|---|---|---|---|---|---|
| | | h | counts min | | |
| $H_2O$ | – | – | – | – | 296250 |
| Lens + globin mRNAs | – | | 14950 | 9750 | 245118 |
| Lens mRNA | $H_2O$ | 4 | 300 | 21300 | 219100 |
| Lens mRNA | globin mRNA | 4 | 19500 | 25000 | 292250 |
| Lens mRNA | $H_2O$ | 22 | 175 | 37300 | 280500 |
| Lens mRNA | globin mRNA | 22 | 4700 | 33900 | 271900 |

mained constant during competition with globin mRNA (Fig 4)

*One Exogenous mRNA versus Endogenous mRNA plus a Second Exogenous mRNA* Exogenous mRNA decreases the rate of translation of endogenous mRNA (Fig 3A and E) It also decreases the translation of a second exogenous mRNA (Fig 3B and F) However, it appears that translation of exogenous mRNAs is inhibited to a greater extent than the translation of endogenous mRNAs This is illustrated most clearly in the experiment depicted in Fig 3C In this experiment the ratio globin endogenous protein is decreased as a result of the cotranslation of increasing amounts of lens mRNA It thus appears that the microinjected mRNAs are more sensitive to competitive inhibition than the endogenous mRNAs

*Subsequent Injection of Two mRNA Species*

If a specific factor limits protein synthesis in oocytes, microinjected mRNAs probably have to acquire it in order to become translated As judged from the time course of translation (Fig 1) this process, perhaps influenced by physical parameters (diffusion?) takes about 6 h While the rate of translation of the microinjected lens mRNA increases, its sensitivity to competitive inhibition by globin mRNA injected afterwards decreases gradually (Fig 5B) Initially translation of lens mRNAs is inhibited much more by globin mRNA than the translation of endogenous mRNAs When the globin mRNA is injected 6 h or more after the lens mRNA, crystallin synthesis and endogenous protein synthesis are inhibited to an equal extent Generally, the second mRNA (globin mRNA) was less efficiently translated when injected long after the first mRNA (lens mRNA) (Table 1) From these double-injection experiments, it appears that the initial discrimination between endogenous and exogenous mRNAs is not a discrimination between amphibian and mammalian mRNAs, but rather between mRNAs already translated and mRNAs yet to be translated After establishment of the injected mRNAs and their incorporation in the protein synthesizing machinery of the oocyte they have acquired the status of active endogenous mRNAs

*Compartmentation of Oocyte mRNAs*

More than 90 % of the polyadenylated mRNA of fully grown *Xenopus* oocytes is found associated with proteins in the postribosomal supernatant, while less than 10 % is engaged in protein synthesis [15] This store of mRNA is apparently mobilized during embryonic development which may proceed up to the gastrula stage in absence of additional RNA synthesis, while protein synthesis increases several fold during this period [16] If the subcellular distribution of injected mRNA molecules (after establishment of the equilibrium) were to be similar to that of the endogenous mRNAs of the oocyte, likewise only about 10 % of the injected mRNA would be incorporated into polyribosomes and be translated However, the translational efficiency (expressed as polypeptides $h^{-1}$ mRNA molecule) of several mRNA species injected in oocytes, among them rabbit globin mRNA [1,5] calf $\alpha A_2$-crystallin mRNA [5] and chicken ovalbumin mRNA [17], was found to be very high In fact, the efficiency of translation in oocytes was close to the efficiency observed in the cell type of origin of these mRNAs at reduced temperature [1,17] (oocytes are incubated at 19 °C) The latter observations are most readily explained by assuming that a large part of the oocyte s endogenous mRNAs are not competing for the limited capacity of the translational apparatus, because they are present in the cytoplasm in a form which in some way is temporarily inactivated [15,18] The outcome of the type of competition experiments described above should then be interpreted as resulting from a competition of the injected mRNA with a restricted subclass of oocyte mRNAs which are avail-

able for translation. Variations in the size of the pool of competing endogenous mRNAs between different batches of oocytes could explain the difference between 'good' and 'bad' oocytes.

We thank Miss M. Koopmans for skilful technical assistance. We are also grateful to Dr. H. P. J. Bloemers for critical reading of the manuscript. These investigations have partly been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).

## REFERENCES

1 Gurdon, J. B., Lane, C. D., Woodland, H. R., & Marbaix, G. (1971) *Nature (Lond.), 233*, 177–182
2 Lane, C. D., Gregory, C. M., & Morel, C. (1973) *Eur. J. Biochem. 34*, 219–227
3 Laskey, R. A., Mills, A. D., Gurdon, J. B., & Partington, G. A. (1977) *Cell 11*, 345–351
4 Berns, A. J. M., van Kraaikamp, M., Bloemendal, H., & Lane, C. D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1606–1609
5 Asselbergs, F. A. M., van Venrooij, W. J., & Bloemendal, H. (1978) *Eur. J. Biochem. 87*, 517–524
6 Lingrel, J. B., & Woodland, H. R. (1974) *Eur. J. Biochem. 47*, 47–56
7 Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M., & Littauer, U. Z. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3143–3146
8 Berridge, M. V., & Lane, C. D. (1976) *Cell 8*, 283–297
9 Berns, A., Jansen, P., & Bloemendal, H. (1974) *Biochem. Biophys. Res. Commun. 59*, 1157–1164
10 Bloemendal, H. (1977) *Science (Wash. D.C.), 197*, 127–138
11 Ghiloni, B., Gianni, A. M., Comi, P., Ottolenghi, S., & Rungger, D. (1973) *Nat. New Biol. 246*, 99–102
12 Moar, V. A., Gurdon, J. B., Lane, C. D., & Marbaix, G. (1971) *J. Mol. Biol. 61*, 93–103
13 Asselbergs, F. A. M., Peters, W. H. M., van Venrooij, W. J., & Bloemendal, H. (1978) *Biochim. Biophys. Acta 520*, 577–587
14 Phillips, J. A., Snyder, P. G., & Kazazian, H. H. Jr. (1977) *Nature (Lond.) 269*, 442–445
15 Rosbash, M., & Ford, P. J. (1974) *J. Mol. Biol. 85*, 87–101
16 Davidson, I. H. (1976) *Gene Activity in Early Development*, 2nd edn., pp. 49–51, Academic Press, New York
17 Chan, L., Kohler, P. O., & O'Malley, B. (1976) *J. Clin. Invest. 57*, 576–585
18 Adamson, F. D., & Woodland, H. R. (1977) *Dev. Biol. 57*, 136–149

F. A. M. Asselbergs, Cold Spring Harbor Laboratory of Quantitative Biology, P.O. Box 100, Cold Spring Harbor, Long Island, New York, U.S.A. 11724

W. J. van Venrooij* and H. Bloemendal, Laboratorium voor Biochemie, Katholieke Universiteit Nijmegen, Geert Grooteplein Noord 21, NL-6525 EZ Nijmegen, The Netherlands

* To whom correspondence should be addressed

Volume 94 number 2 FEBS LETTERS October 1978

# CAP ANALOGUES DO NOT INHIBIT mRNA TRANSLATION IN *XENOPUS LAEVIS* OOCYTES

Fred A M ASSELBERGS, Wilbert H M PETERS, Walther J VAN VENROOIJ and Hans BLOEMENDAL
*Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen, The Netherlands*

Received 12 June 1978
Revised version received 2 August 1978

## 1 Introduction

$m^7G5'ppp5'N$, the cap of eukaryotic mRNA is thought to serve a dual function. It protects the mRNA against 5' exonucleolytic degradation [1,2] and it plays a role during the initiation of protein synthesis (reviewed [3]). Protection against nucleases has been demonstrated using microinjection into *Xenopus laevis* oocytes [1], which most closely resembles in vivo mRNA translation. On the other hand, requirement of the cap for initiation of protein synthesis has only been demonstrated in vitro [3]. However, in some cell free systems the requirement of the cap is not absolute [4-7] and it has, therefore, been suggested that the cap only facilitates initiation of protein synthesis.

In this study an attempt was made to block initiation of protein synthesis in *Xenopus* oocytes by microinjection of cap analogues into the cell. Cap analogues can specifically inhibit translation of cap bearing mRNAs in a wide variety of cell free systems [8-13], provided that the experiments are performed at physiological concentrations of $K^+$ [14,15]. However, in oocytes endogenous protein synthesis and translation of exogenous mRNA were unaffected by the presence of cap analogues.

## 2 Materials and methods

### 2.1 *Materials*

Cap analogues, $m^7G5'p$ and $m^7G5'ppp5'G$, were products from P-L Biochemicals, Milwaukee. Molarity of $m^7G5'ppp5'G$ was calculated from the absorbance assuming $\epsilon_{260} = 20 \times 10^3$ [6]. [$^{35}$S]Methionine (380 Ci/mmol) was purchased from the Radiochemical Centre, Amersham.

### 2.2 *Translation of mRNA in* Xenopus *oocytes*

Isolation of poly(A)$^+$ mRNA from calf lenses and of rabbit globin mRNA has been described [14]. Alfalfa mosaic virus RNA-4 was a generous gift of Dr L. van Vloten Doting (State University of Leiden). Microinjection of mRNAs into *Xenopus laevis* oocytes and subsequent quantitation of the translation products have been described [16].

## 3 Results

### 3.1 *Effect of $m^7G5'p$ on oocyte protein synthesis*

At first an attempt was made to block initiation of endogenous protein synthesis of oocytes by injecting increasing volumes of an $m^7G5'p$ solution (fig. 1). However, the interpretation of this type of experiment was complicated by a stimulation of the incorporation of radioactive methionine into oocyte proteins by the mere injection of fluid (fig. 1A). This phenomenon was not restricted to methionine, but was also observed when other amino acids (leucine, histidine or a mixture of 15 amino acids) were used as precursor. Apparently the effect is primarily due to an increased uptake of radioactive amino acids by the oocytes, in particular since incorporation into protein, when expressed as % radioactivity present in oocytes, is stimulated much less (fig. 1B).

The injections resulted in final $m^7G5'p \leqslant 1.5$ mM inside the living oocyte. However, no significant

*Elsevier/North Holland Biomedical Press*

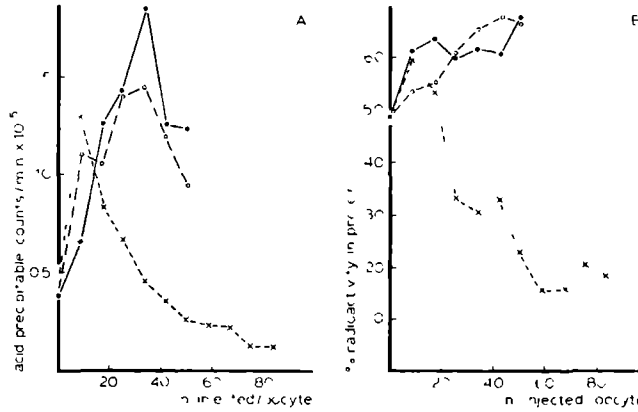



Fig 1 Influence of $m^7G5'p$ and aurintricarboxylic acid on *Xenopus* oocyte protein synthesis (A) Trichloroacetic acid precipitable radioactivity (after alkaline stripping of tRNA) of oocytes injected with increasing volumes of water (● ●) 30 mM $m^7G5'p$ (○- - ○) and 20 mM aurintricarboxylic acid (× ×) (B) Acid precipitable radioactivity of oocytes expressed as % of total (i e acid soluble plus insoluble) radioactivity in order to correct for variations in the uptake of radioactive amino acid from the medium Oocytes were labeled for 6 h immediately after injection

inhibition of oocyte protein synthesis was observed, in contrast to the inhibition observed after injection of aurintricarboxylic acid (fig 1B)

### 3 2 *Effect of cap analogues on translation of injected mRNAs*

In a second series of experiments $m^7G5'p$ was injected together with calf lens mRNA The synthesis of lens proteins (crystallins) can be measured very accurately by quantitative immunoprecipitation [16] In these experiments the injection volume was kept constant However, with 4 mM $m^7G5'p$, there was no obvious inhibition of either total protein synthesis (fig 2A,C) or the synthesis of lens crystallins (fig 2B,D) Also with the larger cap analogue $m^7G5'ppp5'G$, which inhibits protein synthesis in cell-free systems at 5 10 times lower concentrations than $m^7G5'p$ [8,9,13], no significant inhibition was

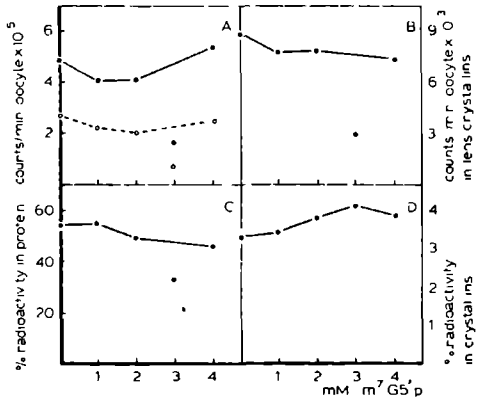



Fig 2 Influence of $m^7G5'p$ on translation of lens mRNAs in *Xenopus* oocytes (A) Oocytes were injected with 25 nl water containing lens mRNA (0 2 mg/ml) and various amounts of $m^7G5'p$ and were labeled for 4 h immediately after injection Intracellular concentration of the cap analogue was calculated assuming a 1 μl oocyte volume Total (●——●) and acid-precipitable (○- - -○) radioactivity of oocytes was measured (B) Radioactive lens crystallins were quantitated using subsequent immunoprecipitations with antisera against αA-, β- and γ-crystallins respectively The sum of the radioactivity in the 3 immunoprecipitates is depicted in the figure (C) Acid precipitable radioactivity of oocytes was expressed as % total oocyte radioactivity (D) Newly synthesized lens crystallins as % acid-precipitable radioactivity of oocytes

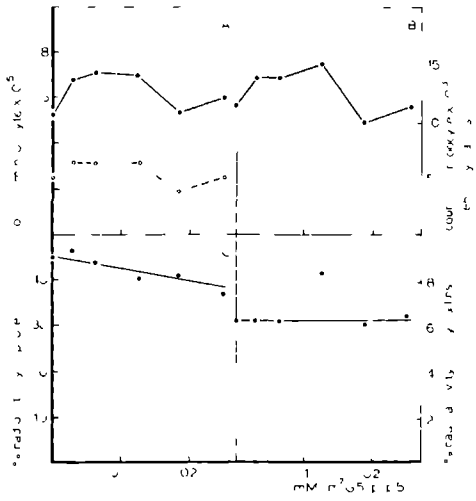


Fig 3 Influence of $m^7G5'ppp5'G$ on translation of lens mRNAs in *Xenopus* oocytes See legend of fig 2 Data were obtained from a different batch of oocytes

observed (fig 3) Inhibition of mRNA translation was also not observed, when any possible leakage of $m^7G5'p$ out of the oocytes was prevented by the presence of an equal concentration of cap analogue in the oocyte culture medium (table 1) Translation of mRNAs coding for rabbit globin and alfalfa mosaic virus coat protein, which is inhibited completely by such concentrations of cap analogues in cell free systems [8 13,17], was in the oocyte unaffected by $m^7G5'p$ (table 1) and $m^7G5'ppp5'G$ (not shown)

## 4 Discussion

The cap analogue concentrations used in this study were sufficient to block the translation of all these mRNAs in an mRNA dependent reticulocyte lysate completely, as shown [13] Further, it has been demonstrated that in an untreated reticulocyte lysate (still containing the endogenous globin mRNA) α crystallin synthesis was ~80% inhibited by 4 mM $m^7G5'p$ and globin synthesis ~60% inhibited [18] In contrast, these concentrations of cap analogues

Table 1
Effect of $m^7G5'p$ on translation of different mRNA species

| Exp | mRNA injected/ oocyte | mM $m^7G5'p$ | Acid precip radioact | | Foreign translation product | |
|---|---|---|---|---|---|---|
| | | | cpm | % total | cpm | % acid precip radioact |
| A | – | 0 0 | 79 270 | 52 0 | | |
| A | – | 1 0 | 81 480 | 51 3 | – | – |
| A | 5 ng lens mRNA | 0 0 | 97 745 | 52 0 | 6590 | 6 74 |
| A | 5 ng lens mRNA | 1 0 | 68 255 | 50 2 | 4220 | 6 48 |
| A | 5 ng globin mRNA | 0 0 | 87 290 | 55 8 | 2890 | 3 30 |
| A | 5 ng globin mRNA | 1 0 | 71 970 | 53 5 | 2070 | 2 88 |
| B | 25 ng AMV RNA 4 | 0 0 | 73 655 | n d | 18 265 | 24 8 |
| B | 25 ng AMV RNA 4 | 1 0 | 55 815 | n d | 14 125 | 25 3 |
| C | 5 ng globin mRNA | 0 0 | 148 930 | n d | 22 575 | 15 2 |
| C | 5 ng globin mRNA | 5 0 | 151 598 | n d | 24 560 | 16 2 |

Water, 25 nl, or mRNA solution, sometimes also containing $m^7G5'p$, was injected into oocytes which were then labeled for 6 h with [$^{35}$S]methionine (exp A,B) or [$^3$H]histidine (exp C) Synthesis of lens crystallins was assayed as in fig 2 legend and synthesis of rabbit globin and alfalfa mosaic virus (AMV) coat protein was measured by determination of the radioactivity present in the appropriate region of an SDS–polyacrylamide gel [16] Intracellular concentration of $m^7G5'p$ was calculated assuming an oocyte vol 1 μl In exp A C $m^7G5'p$ was also included in the oocyte culture medium at the supposed intracellular concentration to neutralize possible diffusion of cap analogue out of the oocytes

do not affect the translation of either exogenous or endogenous mRNA inside living oocytes. Aurintricarboxylic acid, which inhibits the initiation of protein synthesis at low concentrations in vitro and at higher concentrations elongation also [19], strongly inhibited oocyte protein synthesis (fig. 1). However, from our experiments it can not be concluded at which step oocyte protein synthesis in vivo is inhibited.

One could make a number of speculations as to why no inhibition was observed. For instance, it can not be excluded that the cap analogues are degraded rapidly in the oocytes. Another possibility is that even more cap analogue is needed to inhibit translation in oocytes. It should be noted that as a rule more cap analogue is needed to inhibit translation in a cell free system when translation of mRNA is more efficient. Since *Xenopus* oocytes perform translation with the supreme efficiency of a living cell, the insensitivity to cap analogues might simply be a consequence of this efficiency. In the nuclease treated reticulocyte lysate some mRNA apparently escapes inhibition during prolonged incubation and reinitiation of protein synthesis is less inhibited by cap analogues [18]. Likewise mRNAs microinjected into living oocytes might have better chances of escaping the initial inhibition by the cap analogues and the greater translational efficiency will then cause a frequent reinitiation of translation.

## Acknowledgements

The present investigations have been carried out partly under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid of the Netherlands Organization for the Advancement of Pure Research (ZWO).

## References

[1] Uruichi Y., Efstratiadis A. and Shatkin A. J. (1977) Nature 266, 235–239.
[2] Shimotohno K., Kodama Y., Hashimoto J. and Miura K. I. (1977) Proc. Natl. Acad. Sci. USA 74, 2734–2738.
[3] Shatkin A. J. (1976) Cell 9, 645–653.
[4] Rose J. K. and Lodish H. F. (1976) Nature 262, 32–37.
[5] Shih D. S., Distefano R. and Kaesberg P. (1976) J. Virol. 19, 637–642.
[6] Lodish H. F. and Rose J. K. (1977) J. Biol. Chem. 252, 1181–1186.
[7] Held W. A., West K. and Gallagher T. J. (1977) J. Biol. Chem. 252, 8459–8497.
[8] Cantoni D., Revel M. and Groner Y. (1976) FEBS Lett. 64, 326–331.
[9] Groner Y., Crosfeld H. and Littauer U. Z. (1976) Eur. J. Biochem. 71, 281–293.
[10] Hickey E. D., Weber L. A. and Baglioni C. (1976) Proc. Natl. Acad. Sci. USA 73, 19–23.
[11] Shatkin A. J., Weinstein I. A., Sonenberg B., Merrick W. C., Weber L. A., Hickey E. D. and Baglioni C. (1976) Nature 261, 291–294.
[12] Weber L. A., Feigin L. R., Hickey E. D., Williams M. C. and Baglioni C. (1976) J. Biol. Chem. 251, 5657–5662.
[13] Asselbergs F. A. M., Peters W. H. M., Van Venrooij W. J. and Bloemendal H. (1978) Biochim. Biophys. Acta 520, 577–587.
[14] Weber L. A., Hickey E. D., Nuss D. L. and Baglioni C. (1977) Proc. Natl. Acad. Sci. USA 74, 3254–3258.
[15] Kemper B. and Stollorsky T. (1977) Biochemistry 16, 5770–5775.
[16] Asselbergs F. A. M., Van Venrooij W. J. and Bloemendal H. (1978) Eur. J. Biochem. 87, 517–524.
[17] Van Venrooij W. J., Sillekens P. T., Bol J., Reinders R. J., Van Bennekom W. P., Castelein A., Bosch L., Marbaix G., Huez G., Hubert E. and Cleuter Y. (1977) in: Nucleic acids in protein synthesis in plants (Borochov E. and Weil J. H. eds) Plenum, New York.
[18] Asselbergs F. A. M., Peters W. J. M., Van Venrooij W. J. and Bloemendal H. (1978) Eur. J. Biochem. 88, 483–488.
[19] Lodish H. F., Housman D. and Jacobsen M. (1971) Biochemistry 10, 2348–2356.

CHAPTER IV

# POST-SYNTHETIC FATE OF THE TRANSLATION PRODUCTS OF MESSENGER RNA MICROINJECTED INTO XENOPUS OOCYTES

## IV - 1 Post-synthetic fate of the translation products of messenger RNA microinjected into Xenopus oocytes

A. Introduction

B. Intracellular localization of heterologous translation products

C. Proteolytic processing of primary translation products

post-synthetic stability of translation products

proteolytic cleavage of secreted polypeptides: pre-proteins

proteolytic cleavage of poly-proteins

D. Post-synthetic additions to the polypeptide chain

glycosylation

phosphorylation

N-acetylation

prolylhydroxylation

E. Post-synthetic assembly of multimeric proteins in oocytes

F. Post-synthetic modifications not performed by oocytes

G. Future applications of the oocyte system

## IV - 2 Post-translational assembly of lens α-crystallin in the reticulocyte lysate and in Xenopus oocytes

IV-1

# POST-SYNTHETIC FATE OF THE TRANSLATION PRODUCTS OF HETEROLOGOUS MESSENGER RNA IN XENOPUS OOCYTES

## A: Introduction

One of the advantages of the Xenopus oocyte system for the translation of heterologous mRNAs in comparison with cell-free systems resides in the fact, that a number of post-synthetic modifications of the polypeptide chains also occur in a correct manner in oocytes, whereas they do not occur in the cell-free system. This article summarizes our present knowledge of the fate of the heterologous translation products in oocytes and attempts to give an outlook to future possibilities of this experimental system. A list of polypeptides known to be modified after synthesis in oocytes is given at the end of this article (table 1).

## B: Intracellular localization of the heterologous translation products

The intracellular fate of a protein is not purely a function of its physical and chemical properties, but is probably also determined by its intracellular localization, as is perhaps best demonstrated by the example of vitellogenin. The yolk proteins of the Xenopus oocyte, lipovitellin and phosvitin, are synthesized in the liver as a precursor polypeptide, vitellogenin, which is transported by the bloodstream to the ovary (reviewed in 1). Normally, vitellogenin is absorbed by the oocyte by pinocytosis and once inside the cell it is cleaved into lipovitellin and phosvitin, which, are then incorporated into the yolk platelets (2). If, however, the normal cellular transport machinery is bypassed by directly injecting vitellogenin into the oocyte cytoplasm, the injected protein is not processed normally, but is degraded to amino acids, which are further metabolized (3). Now, if vitellogenin is

synthesized in the oocyte after injection of liver mRNA, the resulting translation product is processed normally. It is first phosphorylated, probably also glycosylated (the translation product has the same apparent molecular weight as liver vitellogenin), and subsequently cleaved and incorporated into yolk platelets(4). It is reasonable to assume that correct intracellular localization is an essential prerequisite for correct processing of polypeptides. It is also conceivable that the cell is able to eliminate incorrectly localized proteins. Apparently the translation products of injected mRNAs are automatically inserted into the 'correct' subcellular compartment, since most of them are correctly processed (table 1).

Polypeptides that are to be secreted from a cell, normally are transferred across the endoplasmatic reticular membranes, transported to the Golgi-apparatus and included in excretion vesicles, from which the content is then released from the cell by exocytosis (5). In oocytes injected with the encoding mRNAs, export proteins, like Xenopus vitellogenin, Xenopus albumin and guinea pig milk proteins, are inserted into membrane vesicles, whereas not-to-be-secreted polypeptides, like globin, have never been found in such vesicles (6). If albumin instead of its mRNA, is injected into oocytes it is not found in vesicles (6). It thus seems that the insertion into the vesicles is somehow connected with the synthesis of the polypeptide chain (7).

C: Proteolytic processing of heterologous translation products in oocytes

Post-synthetic stability of translation products

Heterologous products of microinjected mRNAs are fairly stable in Xenopus oocytes. Globin synthesized in oocytes appeared to be

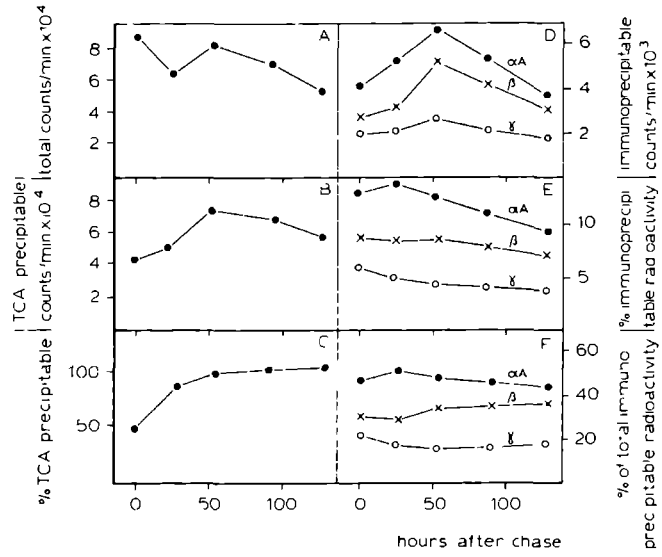



fig. 1 Post-synthetic stability of the lens crystallins in Xenopus laevis oocytes.

Oocytes were injected with 1.3 $OD_{260}$ of lens polyribosomes, pulse labeled for 18 h with ($^{14}C$) protein hydrolysate, transferred into medium containing unlabeled amino acids and incubated for the indicated chase period. Translation products from batches of 10 oocytes were quantitated as previously described (9). (A) Total (i.e. acid-soluble plus - insoluble) radioactivity of oocytes. (B) Trichloroacetic acid (TCA) precipitable radioactivity. (C) Trichloroacetic acid precipitable radioactivity expressed as percentage of the total. (D) Radioactivity quantitatively immunoprecipitated with antisera against αA-(●——●), β-(x----x) and γ-crystallin (o----o), respectively. (E) Immunoprecipitable radioactivity expressed as a percentage of the total trichloroacetic acid precipitable radioactivity. (F) Radioactivity immunoprecipitable with antisera against αA-, β- and γ-crystallin expressed as a percentage of the total immunoprecipitable radioactivity. All values have been normalized to one-oocyte equivalents.

completely stable over a period of 18 h. (8). In a similar pulse-chase experiment the relative amount of the calf lens crystallins measured by quantitative immunoprecipitation (9) dropped from 28% to 21% of the total oocyte protein over a period of five days (fig. 1). In these experiments a slow but definite turnover of the newly synthesized oocyte proteins was observed as well. In another study it was found that the average half life of the oocyte endogenous proteins is 73 h and that, as in most cell types, large polypeptides are degraded faster than small ones (10). The translation products of the viral RNA of several retroviruses (listed in table 1) are generally as stable as the oocyte's own proteins (Asselbergs, unpublished). The observation, that one of the viral proteins of Rauscher murine leukemia virus, p $30^{gag}$, after being stable for 3 days, was completely degraded after 5 days, whereas two other viral polypeptides, p. $15^{gag}$ and p. $12^{gag}$, were not degraded, indicates that selective degradation may occur in oocytes (11). In a pulse-chase experiment with oocytes microinjected with adenovirus mRNA only 20% of the 72 Kdalton single-strand - DNA - binding protein could be immunoprecipitated after a 24 h chase period, while other virus-specific polypeptides appeared to be completely stable. The ssDNA-binding protein appeared to be normally phosphorylated by the oocytes (F. Asselbergs, M. Salden and A. van der Eb, unpublished).

Proteolytic processing of secreted polypeptides: pre-proteins

The findings mentioned in the paragraph above implicate that the signal indicating that the polypeptide must be transferred across the reticular membrane, is also encoded by the mRNA. It has been shown that the $NH_2$-terminal extremity of the export proteins which is rich in hydrophobic amino acids, is recognized by a tunnel-like receptor in the

reticular membrane, as soon as it protrudes from the ribosome (7). The growing polypeptide chain is then pushed through the membrane and as a consequence the growing polypeptide chain anchors the (poly-) ribosome to the endoplasmatic reticulum. In most cases, the $NH_2$-terminal 'signal-peptide' is clipped off very soon after the transfer across the membrane. Even very shortly after synthesis virtually no proteins with 'signal-peptide (mostly referred to as pre-proteins) are found (5). In contrast, 'pre-proteins' can be synthesized in cell-free systems which are devoid of membranes (5).

Processing of pre-proteins in oocytes has been demonstrated for immunoglobulin G, (12) uteroglobin (13, 14), pro-insulin (15), promitellin (16, 17) and possibly the α-subunit of thyrotropin (18) (see also table 1).

Maturation of many polypeptide hormones and some other export proteins as well involves a second proteolytic cleavage after the removal of the pre-piece. This second cleavage occurs in most cells when the polypeptides enter the Golgi-apparatus (5). However, such a second cleavage, for example the conversion of pro-insulin to insulin (15) or of promitellin to mitellin (16, 17) has never been observed in oocytes.

Proteolytic processing of poly-proteins

It is part of the replication strategy of many (especially RNA) viruses, that the initial translation product of the viral RNA is a large precursor-polypeptide, from which by multiple proteolytic cleavages several mature viral proteins are derived (19). Some non-viral proteins, such as corticotropin, lipotropin and β-endorphin (20 and references therein), are also synthesized as parts of a single precursor

polypeptide. The example of the oocyte yolk proteins has already been mentioned above (1-4). Polyproteins of two classes of viruses, picornaviruses and retroviruses, have been shown to be processed in oocytes (table 1).

Picornaviruses:

Compared to the rate of processing of to be exported pre-proteins, which are completely processed within 5 minutes after synthesis (5), the rate of processing of picornavirus polyproteins is slow. The precursors, detected in infected cells, have half-lives of 5-10 minutes (21). Processing of the precursor polypeptides of encephalomyocarditis virus (EMC) has been demonstrated in oocytes (22, 23). 19 h after injection of the viral RNA some but not all of the virion proteins were detected. Some of the final cleavages, which normally occur during assembly of the virion, do also not oocur in a cell-free system programmed with poliovirus RNA (24). Probably, EMC viral RNA can direct the synthesis of its own processing enzyme(s) in a cell-free system (25, 26).

Retroviruses:

The RNA-genome of non-defective type C retroviruses contains three cistrons, 5'-gag-pol-env-3' (reviews, 27-29). The gag-gene encodes four viral structural proteins, which occur in the core of the virus particle and which are called the group specific antigen (gag-) proteins. They are initially synthesized as one precursor polyprotein. A genome size virusspecific mRNA, isolated from polyribosomes of infected cells directs the synthesis of the gag-precursor (30, 31). The virion RNA is also able to direct the synthesis of the gag-precursor in heterologous translation systems (27, 32, 33). The product of the pol-gene, the

viral RNA-dependent DNA-polymerase or reverse transcriptase, also occurs in the virus core, but in very low amounts. It appears to be synthesized as an elongated gag-precursor that contains at the COOH-terminal side the reverse transcriptase, which is cleaved off during subsequent processing (34, 35). The elongation of the gag-precursor may be due to a low frequency of read-through of the stop codon(s) at the end of the gag-coding sequence by the ribosome (33, 36, 37). Alternatively the gag-pol polypeptide may be synthesized from an RNA from which the stop-codon(s) has been eliminated by "splicing" (38). In the latter case it has to be assumed that the gag-pol mRNA is frequently encapsulated into virions, since the gag-pol polypeptide can be synthesized in vitro by translation of the viral RNA (33, 36-39). The envelope (env-) proteins are synthesized by a subgenomic mRNA containing primarily sequences from the 3' end of the viral genomic RNA (40). This subgenomic RNA is not encapsulated into virions (30, 42, 42). The envelope glycoprotein and the smaller non-glycosylated envelope protein are synthesized as one precursor polyprotein.

In Xenopus oocytes micro-injected with RNA from several avian and mammalian retroviruses the gag-precursor is correctly and completely processed into the four viral core proteins (table 1). Such a processing does not occur in most cell-free systems (33, 36-39). The avian myeloblastosis virus gag-precursor has a half-life of about 16 h in oocytes (43) contrasting with the half-life of 45 min observed in infected chicken fibroblasts (44). This slower processing rate in oocytes as compared to the rate in the normal environment is also observed when RNA of several mammalian retroviruses is injected into oocytes (11, 43, 45-47; Asselbergs and Salden, unpublished) and it may be a consequence of the lower incubation temperature (19$^{o}$C) of Xenopus oocytes (43).

Genetic evidence suggests that just as in processing of the polyproteins of picornaviruses (25, 26, 48) and togaviruses (49) the processing of the gag-precursor is partially specified by viral gene products (50). The gag-precursor of Rous (avian) sarcoma virus can be cleaved in vitro by one of its cleavage products, the viral core protein $p15^{gag}$ (51, 52). However, this cannot completely explain the correct processing of the gag-precursors of retroviruses in oocytes (table 1), since the cleavages proceed, at least initially, in complete absence of any viral translation product. Furthermore, polypeptide sequence data indicate that more than one protease is involved in the processing of the murine leukemia virus gag-precursor, pr $65^{gag}$ (52). In accordance with the concept that both viral and cell coded proteases are involved in processing is the observation of different processing pathways of pr $65^{gag}$ at early and late times after injection of Rauscher murine leukemia viral RNA into oocytes (11). The observation that when an oocyte homogenate containing the radioactive gag-precursor of avian myeloblastosis virus is injected into oocytes, the precursor is not processed unless also the viral RNA is injected (J. Ghysdael, personal communication) also suggests that continuous production of a virus-specific factor is required for processing of the gag-polyprotein.

In cells infected with murine leukemia viruses two types of gag-precursor, pr $76^{gag}$ and pr $65^{gag}$, are found (fig. 2) (35, 53). The smaller type, pr $65^{gag}$, contains only the four gag-proteins and, therefore, may be regarded to be the equivalent of the gag-precursor of avian leukemia virus, (fig. 2) in which the primary translation product of the gag-gene contains only the four gag-proteins (29). Despite the similarity

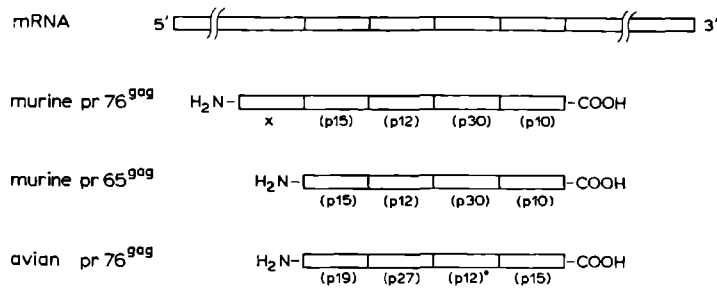



fig. 2 Arrangements of viral proteins within the murine and avian gag-gene-coded precursor-polypeptides.

The values between bracket indicate the molecular weights of individual viral proteins as determined by agarose gel filtration in the presence of 6M guanidine-HCl. Values are given for Rauscher murine leukemia virus and avian myeloblastosis virus. The arrangement of homologous proteins within the murine and avian precursor is not the same. Murine p15 is equivalent to avian p15 (internal hydrophobic protein), murine p12 is equivalent to avian p19 (internal, major phosphoprotein, specific RNA binding properties), murine p30 is equivalent to avian p27 (internal, most abundant protein) and murine p10 is equivalent to avian p12 (internal, ribonucleoprotein). (26) The $NH_2$-terminal polypeptide x is not found in the primary translation product of the avian gag-gene, $pr76^{gag}$ (83).

* The position of p12 within the avian gag-precursor is not entirely certain. It may be at either side of p27, but the available data favor a position between p27 and p15 (44).

of these precursors it should be kept in mind that the order of the different gag-proteins within the precursor is different in avian and murine viruses (28). Pr $76^{gag}$ and pr $65^{gag}$ appear to be synthesized by separate 35 S mRNAs, since (a) both gag-precursors can be labeled with N-formylmethionyl-$tRNA_i$ in a cell-free system (54) and (b) yeast amber tRNA suppresses termination of pr $76^{gag}$, but not pr $65^{gag}$, thus causing enhanced synthesis of the gag-pol read-through polypeptide (35, 38). In oocytes injected with MuLV viral RNA the rate of synthesis of pr $76^{gag}$ compared to pr $65^{gag}$ is gradually reduced after prolonged culture of the oocytes, which suggests that the pr $76^{gag}$ mRNA may be functionally unstable in oocytes (11). The major gag-precursor (pr $65^{gag}$) is probably the direct precursor of the viral core proteins (27-29). The minor gag-precursor (pr $76^{gag}$) contains at the $NH_2$-terminus an additional polypeptide, here designated 'X' (fig. 2) (55). Perhaps as a consequence of a 'signal' present in this $NH_2$-terminal polypeptide pr $76^{gag}$ appears to be diverted onto a different processing pathway in the cell. It is probably converted to a series of glycosylated gag-polypeptides with molecular weights of 90-95 kdalton, part of which are incorporated into the cell membrane, whereas another part is secreted (54). Such 90-95 kdalton polypeptides have also been detected in oocytes and the kinetics of appearance after injection of the viral RNA suggested that they resulted from a post-translational modification of another gag-precursor, presumably pr $76^{gag}$ (11).

As discussed above, retroviral genomic RNA is able to direct besides the precursor of the viral core polypeptides the synthesis of an even larger precursor that in addition to the core polypeptides contains also

the reverse transcriptase. A metabolically unstable polypeptide, of 170 kdalton which may be identical to this combined precursor, was detected as a minor translation product of avian myeloblastosis viral RNA in oocytes (F. Asselbergs and M. Salden, unpublished results; J. Ghysdael, personal communication). A large translation product (145 kDalton) with similar properties was detected after injection of bovine leukemia virus RNA (47).

A subgenomic mRNA isolated from membrane-bound polyribosomes of retrovirus producing cells directs the synthesis of the polypeptides of the viral envelope (30, 41, 42). Oocytes microinjected with the subgenomic mRNA of Rauscher murine leukemia virus synthesize the glycosylated envelope precursor and process it correctly into the mature glycoprotein gp $70^{env}$ and the non-glycosylated p $15^{env}$ (30).

D. Post-synthetic additions to the polypeptide chain: Glycosylation of heterologous translation products

Glycoproteins are synthesized by the polyribosomes bound to the endoplasmatic reticulum. Attachment of the ribosomes to the membrane by the nascent polypeptide chain probably occurs in a similar manner as with secretory proteins. Sugar residues are incorporated at specific sites, while the polypeptide is still nascent, but the carbohydrate chains may be elongated after intracellular translocation to the Golgi-apparatus (56). The signal in the amino acid sequence of a protein specifying a glycosylation site has apparently been very conservative during evolution, since oocytes correctly glycosylate the translation products from mRNAs of mammals and birds (table 1). Only those immunoglobulins, which normally contain carbohydrates, are glycosylated in

oocytes, while a non glycosylated type of immunoglobulin is also not glycosylated in oocytes (57). Most of the heterologous translation products known to be glycosylated in oocytes (table 1) are secretory proteins (immunoglobulins, (57, 58) interferon (59), ovalbumin (60) and thyrotropin (61)), but the envelope glycoprotein of Rauscher murine leukemia virus synthesized in oocytes after injection of subgenomic virus-specific mRNA isolated from membrane-bound polyribosomes of infected cells is a glycosylated plasma membrane protein (30).

Phosphorylation of heterologous translation products

Oocytes have been shown to phosphorylate the translation products of several microinjected mRNAs encoding phosphoproteins. (table 1). The phosphorylation of vitellogenin and of the precursor of the core proteins of mouse mammary tumor virus (62) preceeds the cleavage of these polyproteins. The single-strand DNA-binding protein of adenovirus and protamine both normally accumulate in the nucleus, but it is not known, whether they also accumulate in the nucleus of oocytes. The phosphorylation of the DNA binding protein takes place over a period of several hours both in oocytes (F. Asselbergs, M. Salden and A. van der Eb, unpublished) and in adenovirus infected cells (63).

N-terminal acetylation of nascent polypeptide chains

Oocytes injected with 14S lens crystallin mRNA have been shown to synthesize exclusively $\alpha A_2$-crystallin in which the N-terminal methionine is acetylated (64). This post-synthetic modification also occurs in the reticulocyte lysate (65), in which it can be prevented by enzymatic removal of coenzyme A from the lysate (66). N-terminal acetylation of $\alpha A_2$-crystallin did not occur in the Krebs II ascites cell-free system, probably because during preincubation and gel filtration the endogenous coenzyme A had been removed (67).

Prolyl-hydroxylation of collagen polypeptides

Proline residues in the translation products of injected mammalian (8) and amphibian collagen mRNAs (68) are efficiently hydroxylated by Xenopus oocytes (table 1). Collagen is a protein exclusively found in animals. An unhydroxylated form of collagen can be obtained by translating collagen mRNA in a plant cell-free system and this can be used as a substrate for the prolylhydroxylase, a soluble enzyme isolated from animal tissue (69).

E. Post-synthetic assembly of multimeric proteins in oocytes

Two types of assembly of heterologous translation products have been demonstrated in oocytes, namely synthesis of polypeptide-complexes held together by S-S-bonds (14, 20, 58, 70-73) and of non-covalently bound complexes (74, 75) (table 1). The S-S-bonds are not formed in cell-free systems (14) probably because these of cell-extracts are made in the presence of a reductive agent, usually dithioerythritol. α-Crystallin, a non-covalently bound complex, is also not assembled in a heterologous cell-free system, presumably because the concentration of monomer polypeptides in this cell-free system is much lower than in the homologous (lens) cell-free system or in oocytes (74).

F. Post-synthetic modifications not performed by Xenopus oocytes

As already mentioned above, oocytes apparently do not perform the type of proteolytic cleavage associated with the terminal phase of secretion, for example the conversion of proinsulin into insulin (14) or promitellin into mitellin (16, 17). Certain types of modifications, which are tissue specific, for example the iodination of thyroglobulin (72), do also not occur in oocytes. The post-synthetic cleavage of the

polypeptide chains of β-glucuronidase, an enzyme present in (mouse) kidney lysosomes, may also be tissue specific since it does not occur in oocytes. Nevertheless, the protein synthesized by the oocytes is enzymatically active (73). Another possible tissue specific modification which does not occur in oocytes is the synthesis of the C-terminal amide of promitellin (16), which probably arises by an enzymatic replacement of the terminal glycine of the primary translation product by ammonia (17).

G. Future applications of the oocyte system

Amphibian oocytes already have proven to be a versatile system for studying the translation of mRNA and the post-synthetic modification of the resulting polypeptides, which surely will find more applications in future experiments. Only two of such applications will be mentioned here. First, the oocyte translation system allows detection of translation products by means of their functional properties. Functional activities of heterologous translation products, which depend on post-synthetic modifications that have been demonstrated in oocytes, include the antigen binding activity of immunoglobulins (82) and the enzymatic activity of β-glucuronidase (73). A second possible application of the oocyte system would be in localizing some of the enzymes performing the post-synthetic modification of particular translation products. For example, it should be possible to find out using normal and enucleated oocytes, whether phosphorylation of nuclear proteins occurs exclusively in the nucleus or in the cytoplasm. In this way the oocyte system would continue to contribute to our understanding of the molecular biology of post-synthetic modifications of polypeptides.

Table 1 POST-SYNTHETIC MODIFICATION OF PRIMARY TRANSLATION PRODUCTS IN OOCYTES

| Modification in normal tissue of origin | Protein | Organ/cell type | Evidence for modification in oocytes | Refs. |
|---|---|---|---|---|
| Proteolytic cleavage of secreted polypeptides | Immunoglobulin light chain precursor | Mouse plasmacytoma cell | Identical mobility as mature light chain during gel electrophoresis 1). | 12 |
| | Pre-uteroglobin (to uterglobin | Rabbit uterus | Identical mobility as mature uteroglobin during gel electrophoresis 1). | 13, 14 |
| | Pre-proinsulin 2) (to proinsulin) | Carp pancreatic islets | Identical mobility as proinsulin during gel electrophoresis 1). | 15 |
| | Pre-promitellin (to promitellin) | Honeybee venom gland | Electrophoretic mobility, partial amino acid sequence. | 16, 17 |
| Proteolytic processing of polyproteins | Vitellogenin (to phosvitin and lipovitellin, incorporated into yolk platelets) | Frog liver | Shift in electrophoretic mobility during pulse-chase experiment. | 4 |

| Modification in normal tissue of origin | Protein | Organ/cell type | Evidence for modification in oocytes | Refs. |
|---|---|---|---|---|
| Proteolytic processing of viral polyproteins | Encephalomyocarditis Virus | Mouse cells | Apparent molecular weight identical to that of mature viral proteins 1). | 22, 23 |
| | Precursor of avian myeloblastosis Virus | Chicken fibroblasts | Apparent molecular weight identical to that of mature viral proteins 1), | 32, 43, 76 |
| | core proteins | Idem | pulse-chase exp. | |
| | Precursor of avian sarcoma virus core proteins | Idem | Apparent molecular weight identical to that of mature viral proteins 1). | 77 |
| | Precursor of Rauscher murine leukemia virus core proteins | Mouse spleen/thymus cells (JLS $V_9$-cells) | Apparent molecular weight identical to that of mature viral proteins 1). | 1, 30, 33, 45 |
| | Idem | Idem | Pulse-chase exp. | 11 |

| Modification in normal tissue of origin | Protein | Organ/cell type | Evidence for modification in oocytes | Refs. |
|---|---|---|---|---|
| Proteolytic processing of viral polyproteins | Precursor of Rauscher murine leukemia virus envelope proteins | Mouse spleen/thymus (JLS-$V_9$cells) | Apparent molecular weights of precursor and mature processing products identical with those from infected cells. | 30 |
| | Precursor of bovine leukemia virus core proteins | Bovine leukemic cell | Apparent molecular weight of mature processing products identical to viral proteins, pulse-chase exp. | 46, 47 |
| | Precursor of mammary tumor virus core proteins | Mouse Mm5mt/cl cells | Apparent molecular weight identical to that of mature viral proteins 1). | 62 |
| Glycosylation of secreted polypeptides | Immunoglobulin heavy chain | Rat spleen cells | Fucose incorporation in immunoprecipitated polypeptide. | 58 |

| Modification in normal tissue of origin | Protein | Organ/cell type | Evidence for modification in oocytes | Refs. |
|---|---|---|---|---|
| Glycosylation of secreted polypeptides | Immunoglobulin light chain | Mouse plasma cytoma | Mannose incorporation into immunoprecipitated polypeptide. | 57 |
| | Interferon | Human fibroblasts | Retention on Con-A-sepharose column, | 59 |
| | Ovalbumin | Chicken oviduct | Incorporation of UDP-N-acetyl-glucosamine | 60 |
| | Thyrotropin α-subunit | Mouse thyrotropic tumor | Identical mobility as native glycosylated hormone 1) 4). | 61 |
| Glycosylation of a membrane protein | Rauscher leukemia virus envelope protein gp 70 | Mouse spleen/thymus cells (JLS-$V_9$ cells) | Identical mobility as native glycoprotein during gel electrophoresis 1). | 30 |
| Phosphorylation | Precursor of mammary tumor virus core proteins | Mouse Mm5mt/cl cells | Apparent molecular weight identical as native phosphorylated precursor. | 62 |
| | Protamine | Trout testis | Phosphorylated peptides. | 78 |
| | Vitellogenin | Frog liver | Phosphorylated polypeptide. | 4 |
| | | Chicken liver | Identical mobility as native (phosphorylated) polypeptide in gel electrophoresis 1). | 79 |

| Modification in normal tissue of origin | Protein | Organ/cell type | Evidence for modification in oocytes | Refs. |
|---|---|---|---|---|
| | Adenovirus 5 72.000 dalton DNA binding protein | HeLa-Cell | Similar increase of apparent molecular weight during pulse-chase exp. as in adenovirus infected cells. 4) | |
| Acetylation of N-terminal methionine | $\alpha A_2$-crystallin | Bovine eye lens | N-acetyl-methionine in N-terminal peptide. | 64 |
| Proline hydroxylation | Collagen | Mouse fibroblast | Hydroxyproline in labeled protein. | 8 |
| | | Frog embryo | Idem. | |
| Assembly of multimeric proteins bound by s-s bonds | Uteroglobin (dimer) | Rabbit uterus | Apparent molecular weight in gel electrophoresis with/without reduction of -S-S-Bonds. | 13, 14 |
| | Immunoglobulin (tetramer) | Plasmacytoma | Apparent molecular weight in gel electrophoresis in absence of reductive agents. | 58, 71<br>70 (cf note 5) |
| | Thyroglobulin (dimer) | Bovine thyroid gland | Ultracentrifugation of 19S protein in absence of reductive agents. | 72 |

| Modification in normal tissue of origin | Protein | Organ/cell type | Evidence for modification in oocytes | Refs. |
|---|---|---|---|---|
| Assembly of multimeric protein with non-covalent bonds between subunits | α-Crystallin (2 types of subunits, about 40 subunits in each protein) | Bovine eye lens | Gel filtration under non-denaturating conditions | 74 |
| | $\beta_L$-crystallin (di- and trimers of different subunits) | Bovine eye lens | Idem. | 75 |
| | β-glucuronidase (tetramer) | Mouse kidney | Mobility in gel electrophoresis under non-denaturing conditions. | 73 |
| Unidentified | Steroid binding protein | Rat prostate | Electrophoretic mobility distinct from translation product from cell-free system, similar to that of native protein. | 80 |

Notes to Table 1

1. This type of evidence is only included in cases where the unmodified polypeptide has a different electrophoretic mobility.

2. A larger product than proinsulin was exclusively synthesized after injection of mRNA from cod pancreatis islets or from a human insulinoma. (77).

3. Part of the translation products appeared to be unglycosylated.

4. Unpublished experiments of the author in collaboration with M.H.L. Salden and A. van der Eb.

5. In this experiment Xenopus eggs were used instead of oocytes.

References

1. Tata, J.R., The expression of the vitellogenin gene, Cell 9, 1-14 (1976).

2. Wallace, R.A. and Jared, D.W., Protein incorporation by isolated amphibian oocytes: specificity for vitellogenin incorporation. J. Cell Biol. 69, 345-351 (1976).

3. Dehn, P.F. and Wallace, R.A., Sequestred and injected vitellogenin; alternative routes of protein processing in Xenopus oocytes. J. Cell Biol. 58, 721-724 (1973).

4. Berridge, M.V. and Lane, C.D. Translation of Xenopus liver mRNA in Xenopus oocytes, vitellogenin synthesis and conversion to yolk platelets. Cell 8, 203-297 (1976).

5. Habener, J.F. and Kennedy, H.M. Parathyroid hormone biosynthesis: structure and function of biosynthetic precursors. Fed. Proc. 37, 2561-2566. (1978).

6. Zehavi-Willner, T. and Lane, C.D., Subcellular compartitation of albumin and globin made in oocytes under the direction of injected messenger RNA. Cell 11, 683-693.

7. Blobel, G. and Dobberstein, B. Transfer of proteins across membranes. J. Cell Biol. 67, 852-862 (1975).

8. Lane, C.D. and Knowland, J.S., The injection of RNA into living cells; the use of frog oocytes for the assay of mRNA and the study of the control of gene expression. in: Biochemistry of development. Vol. 3 (R. Weber, ed.) New York, Academic Press, pp. 145-181 (1975).

9. Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H., Synthesis of lens crystallins in Xenopus oocytes as determined by quantitative immunoprecipitation. Eur. J. Biochem. 8, 517-524. (1978).

10. Wallace, R.A. and Hollinger, T.G., Turnover of endogenous, micro-injected and sequestred proteins in Xenopus oocytes, Exp. Cell Res. 119, 277-287 (1979).

11. Asselbergs, F.A.M., Salden, M.H.L. and Bloemendal, H., Kinetics of synthesis and proteolytic processing of precursor polypeptides of Rauscher Leukemia Virus in frog oocytes following micro-injection of viral RNA. Virology, submitted for publication.

12. Mach, B., Faust, C.F., Vassali, P. and Rungger, D. Different size of the products of the 14S light chain mRNA translated in vitro and in amphibian oocytes. Mol. Biol. Rep. 1, 3-6. (1973)

13. Beato, M. and Rungger, D. Translation of the messenger RNA for rabbit uteroglobin in Xenopus oocytes. FEBS Lett. 59, 305-309, (1975).

14. Beato, M. and Nieto, A., Translation of the mRNA for rabbit uteroglobin in cell-free systems. Eur. J. Biochem. 64, 15-25 (1976).

15. Rapoport, T.A., Thiele, B.J., Prehn, S., Marbaix, G., Cleuter, Y., Hubert, E. and Huez, G., Synthesis of carp proinsulin in Xenopus oocytes. Eur. J. Biochem 87, 229-233 (1978).

16. Kindas-Mugge I., Lane C.D. and Kreil, G., Insect-protein synthesis in frog cells: the translation of honey bee promitellin messenger RNA in Xenopus oocytes. J. Mol. Biol. 87, 451-462 (1974).

17. Kreil, G. Suchanek, G. and Kindas-Mugge, I., Biosynthesis of a secretory polypeptide in honey bee venom glands. Intermediates detected in vivo and in vitro. Fed. Proc. 36, 2081-2088 (1977).

18. Kourides, I.A. and Weintraub, B.D., mRNA-directed biosynthesis of α-submit of thyrotropin: Translation in cell-free and whole-cell systems., Proc. Natl. Acad. Sci. USA 76, 298-302 (1979).

19. Shatkin, A.J., Banerjee, A.K. and Both, G.W., Translation of animal virus RNAs in vitro in: Comprehensive Virology, Vol. 10 (Fraenkel Conrat H. and Wagner R.R., eds) Plenum Press, New York, 1977.

20. Bertagna, X.Y., Nicholson, W.E., Sorenson, G.D., Pettengill, O.S., Mount, C.D. and Orth, D.N., Corticotropin, lipotropin and β-endorphin production by a human nonpituary tumor in culture: Evidence for a common precursor. Proc. Natl. Acad. Sci. USA 75, 5160-5164, (1978).

21. Cooper, P.D., Genetics of Picornaviruses, in: Comprehensive Virology, Vol. 9 (Fraenkel-Conrat H. and Wagner, R.R., eds.) Plenum Press, New York, 1977.

22. Laskey, R.A., Gurdon, J.B. and Crawford, L.V. Translation of encephalomyocarditis virus RNA in oocytes of Xenopus laevis. Proc. Natl. Acad. Sci. USA 69, 3665-3669, (1972).

23. Laskey, R.A., Mills, A.D., Gurdon, J.B. and Partington, G.A. Protein synthesis in oocytes of Xenopus laevis is not regulated by the supply of mRNA. Cell 11, 345-352 (1977).

24. Shih, D.S., Shih, C.T., Kew, O., Pallansch, M., Rueckert, R. and Kaesberg, P. Cell-free synthesis and processing on the proteins of poliovirus. Proc. Natl. Acad. Sci. USA, 75, 5801-5811 (1978).

25. Pelham, H.R.B. Translation of encephalomyocarditis virus in vitro yields an active processing enzyme Eur. J. Biochem. 85, 457-462 (1978).

26. Shih, D.S., Shih, C.T., Zimmern, D., Pueckert, R.R. and Kaesberg, P., Translation of encephalomyocarditis virus RNA in reticulocyte lysates: Kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. 30, 472-480 (1979).

27. Bishop, J.M. Retroviruses, Ann. Rev. Biochem. 47, 35-88 (1978).

28. Eisenman, R.N. and Vogt, V.M., Biosynthesis of oncovirus proteins, Biochim. Biophys. Acta. 473, 188-239 (1978).

29. Stephenson, J.R., Devare, S.G. and Reynolds, F., Translation products of type C RNA tumor viruses, Adv. Cancer Res. 27, 1-54 (1978).

30. Van Zaane, D., Gielkens, A.J.L., Hesselink, W.G. and Bloemers, H.P.J. Identification of Rauscher leukemia virus-specific mRNAs for the synthesis GAG and ENV gene products. Proc. Natl. Acad. Sci. USA. 74, 1855-1859 (1977).

31. Mueller-Lantzsch N and Fan H., Monospecific immunoprecipitation of murine leukemia virus polyribosomes: Identification of p30 specific messenger RNA. Cell 9, 579-585.

32. Salden, M., Asselbergs, F.A.M. and Bloemendal, H. Translation of oncogenic virus RNA in Xenopus laevis oocytes, Nature 259, 696-699 (1976).

33. Salden, M.H.L., Selten-Versteegen, A.M. and Bloemendal, H. Translation of Rauscher murine leukemia viral RNA. A model for the function of a virus-specific messenger. Biochem. Biophys. Res. Commun. 72, 610-618 (1976).

34. Opperman, H., Bishop, J.M., Varmus, H.E. and Levintow, L., A joint product of the genes gag and pol of avian sarcoma virus: a possible precursor of reverse transcriptase Cell 12, 993-1005 (1977).

35. Jamjoom, G.A., Naso, R.B. and Arlinghaus, R.B. (1977) Further characterization of intracellular precursor polyproteins of Rauscher leukemia virus, Virology 78, 11-34.

36. Philipson, L., Anderson, P., Olshevsky, U., Weinberg, R., Baltimore D., and Gesteland, R. Translation of MuLV and MSV RNAs in nuclease treated reticulocyte extracts: enhancement of the gag-pol polypeptide with yeast suppressor tRNA, Cell 13, 189-199.

37. Murphy, E.C., Kopchick, J.J., Watson, K.F. and Arlinghaus, R.B. Cell-free synthesis of a precursor polyprotein containing both gag and pol gene products by Rauscher leukemia virus 35S RNA Cell 13, 359-369.

38. Weiss, S.R., Hackett, P.B., Oppermann, H., Ullrich, A., Levintow, L., and Bishop, J.M., Cell-free translation of avian sarcoma virus RNA: Suppression of the gag termination codon does not augment the synthesis of the joint gag/pol product, Cell 15, 607-614 (1978).

39. Kerr, I.M., Olshevsky, U., Lodish, H.F. and Baltimore, D. Translation of murine leukemia virus RNA in cell-free systems from animal cells. J. Virol 18, 627-635 (1976).

40. Rothenberg, E., Donaoghue, D.J. and Baltimore, D., Analysis of a 5' leader sequence on murine leukemia virus 21S RNA: heteroduplex mapping with long reverse transcriptase products Cell 13, 435-451.

41. Gielkens, A.J.L., van Zaane, D., Bloemers, H.P.J. and Bloemendal, H. Synthesis of Rauscher leukemia virus specific polypeptides in vitro. Proc. Natl. Acad. Sci. USA 73, 356-360 (1976).

42. Stacey, D.W., Allfrey, V.G. and Hanafusa, H. Microinjection analysis of envelope-glycoprotein messenger activities of avian leukosis virus. Proc. Natl. Acad. Sci. USA 74, 1614-1618 (1977).

43. Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D., Burny, A., Cleuter, Y., Huez, G., Kettmann, R., Marbaix, G., Portetelle, D., and Chantrenne, H. Frog oocytes synthesize and completely process the precursor polypeptide to virion structural proteins after microinjection of avian myeloblastosis virus RNA. Proc. Natl. Acad. Sci., USA., 74, 3230-3234 (1977).

44. Vogt, V.M., Eisenman, R. and Diggelman, H. Generation of avian myeloblastosis virus structural proteins by proteolytical cleavage of a precursor polypeptide. J. Mol. Biol. 96, 471-493 (1975).

45. Reynolds, R.K., Van de Ven W.J.M. and Stephenson, J.R. Translation of type C viral RNAs in Xenopus oocytes; Evidence that the 120,000 molecular weight polyprotein expressed in Abelson Leukemia virus transformed cells is viral coded. J. Virol. 25, 665-670 (1978).

46. Burny, A., Bex, F., Chantrenne, H., Cleuter, Y., Dekegel, D., Ghysdael, J., Rettmann, R., Leclercq, M., Leunen, J., Mammerick, M. and Portetelle, D. Bovine leukemia virus involvement in endozootic bovine leukemia. Advances in Cencer Res. Vol 28, (Klein, G., and Weinhouse, S., eds.) Acad. Press, New York, pp. 252-312, (1978).

47. Ghysdael, J., Kettmann, R., and Burny, A., Translation of bovine leukemia virus RNAs in heterologous protein synthesizing systems J. Virology, 29, 1987-1098 (1979).

48. Esteban, M. and Kerr, I.M., The synthesis of encephalomyocarditis virus polypeptides in infected L-cells and cell-free systems. Eur. J. Biochem. 45, 567-576 (1974).

49. Scupham, R.K., Jones, K.J., Sagik, B.P. and Bose, H.R. Virus directed post-translational cleavage in Sindbis virus-infected cells. J. Virol 22, 568-571 (1977).

50. Sacks, T.L., Devare, S.G., Blennerhasset, G.T. and Stephenson, J.R. Nonconditional replication mutants of type C and D retroviruses defective in gag gene-coded post-translational processing Virology 91, 352-363 (1978).

51. Von der Helm, K. Cleavage of Rous sarcoma viral polypeptide precursor to internal structural proteins in vitro involves viral protein p. 15. Proc. Natl. Acad. Sci. USA., 74, 911-915 (1977).

52. Oroszlan, S., Henderson, L.E., Stephenson, J.R., Copeland, T.D., Long, C.W., Ihle, J.N., and Gilden, R.V. Amino and carboxyl-terminal amino acid sequences of proteins coded by gag and gene of murine leukemia virus. Proc. Natl. Acad. Sci. USA., 75, 1404-1408 (1978).

53. Van Zaane, D., Dekker-Michielsen, M.J.A. and Bloemers, H.P.J. Virus-specific precursor polypeptides in cells infected with Rauscher leukemia virus: synthesis, identification and processing Virology 75, 113-129.

54. Edwards, S.A. and Fan, H., Gag-related polyproteins of Moloney murine leukemia virus: Evidence for independent synthesis of glycosylated and unglycosylated forms. J. Virol. 30, 551-563 (1979).

55. Schultz, A.M. and Oroszlan, S., Murine leukemia virus gag polyproteins: The peptide chain unique to pr 80 is located at the amino terminus, Virology 91, 481-486 (1978).

56. Rothman, J.E., Katz, F.N. and Lodish, H.F. Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. Cell 15, 1447-1454 (1978).

57. Jilka, R.L., Cavalieri, R.L., Yaffe, L. and Pestka, S. Synthesis and glycosylation of the MOPC-46B immunoglobulin kappa chain. Biochem. Biophys. Res. Commun. 79, 625-630 (1977).

58. Deacon, N.J. and Erbinger, A. Fucose incorporation into oocyte synthesized rat immunoglobin, FEBS 79, 191-194 (1977).

59. Reynolds, F.H., Jr. Premkumar, E. and Pitha, P.M. Interferon activity produced by translation of human interferon messenger RNA in cell-free system and in Xenopus oocytes. Proc. Natl. Acad. Sci. USA 72, 4881-4885 (1975).

60. Chan, L., Kohler, P.O. and O'Malley, B.W. Translation of ovalbumin mRNA in Xenopus laevis oocytes. Characterization of the system and effects of estrogen on injected mRNA populations. J. Clin. Invest. 57, 576-585 (1976).

61. Kourides, I.A. and Weintraub, B.D. mRNA-directed biosynthesis of α-submit of thyrotropin: Translation in cell-free and whole-cell systems., Proc. Natl. Acad. Sci. USA 76, 298-302 (1979).

62. Nusse, R., Asselbergs, F.A.M., Salden, M.H.L., Michalides, R.J.A.M., and Bloemendal, H., Translation of mouse mammary tumor virus RNA; Precursor polypeptides are phosphorylated during processing., Virology 21, 106-115 (1978).

63. Axelrod, N., Phosphoproteins of adenovirus 2, Virology 87, 366-383, (1978).

64. Berns, A.J.M., van Kraaikamp, M., Bloemendal, H. and Lane, C.D. Calf crystallin in frog cells: The translation of lens cell 14-S mRNA in oocytes. Proc. Natl. Acad. Sci. USA 69, 1606-1609 (1972).

65. Berns, A.J.M, Strous, G.J.A.M., and Bloemendal, H. Heterologous in vitro synthesis of lens α-crystallin polypeptide. Nature New Biology 235, 7-9, (1972).

66. Palmiter, R., Prevention of $NH_2$-terminal acetylation of proteins synthesized in cell-free systems. J. Biol. Chem. 252, 8781-8783 (1977).

67. Berns, A.J.M., Thesis, University of Nijmegen, Nijmegen, The Netherlands. (1972).

68. Rollins, J.W. and Flickinger, R.A., Collagen synthesis in Xenopus oocytes after injected of nuclear RNA in frog embryos. Science 178, 1204-1205 (1972).

69. Beneviste, K., Wilczek, J., Ruggieri, A., and Stern, R., Translation of collagen messenger RNA in a cell-free system derived from wheat germ. Biochemistry 15, 830-835.

70. Stevens, R.H. and Williamson, A.R., Specific mRNA molecules from myeloma cells in heterogeneous nuclear and cytoplasmic RNA containing poly-A. Nature 239, 143-146 (1972).

71. Stevens, R.H. and Williamson, A.R., Translational control of immunoglobulin synthesis: II Cell-free interaction of myeloma immunoglobulin with RNA. J. Mol. Biol. 78, 517-525 (1978).

72. Vassart, G., Refetoff, S., Brocas, H., Dinsart, C. and Dumont, J.E., Translation of thyroglobulin 33S messenger RNA as a means of determining thyroglobulin quaternary structure. Proc. Natl. Acad. Sci. USA. 74, 4462-4465 (1977).

73. Labarca, C. and Paigen, K., mRNA - directed synthesis of catalytically active mouse B-glucuronidase in Xenopus oocytes. Proc. Natl. Acad. Sci. USA 74, 4462-4465 (1977).

74. Asselbergs, F.A.M., Koopmans M., van Venrooij, W.J. and Bloemendal, H., Post-translational assembly of lens α-crystallin in the reticulocyte lysate and in Xenopus laevis oocytes. Eur. J. Biochem. 91, 65-78 (1978).

75. Asselbergs, F.A.M., Koopmans, M., van Venrooij, W.J. and Bloemendal, H., β-crystallin in Xenopus oocytes. Exp. Eye Res., in press.

76. Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D.P., Cleuter, Y., Huez, G., Marbaix, G., Portetelle, D. and Chantrenne, H. Synthesis and complete processing of a high-molecular-weight precursor polypeptide to viral structural proteins in toad oocytes microinjected with avian myeloblastosis virus ribonucleic acid. Biochem. Soc. Trans. 5, 950-953 (1977).

77. Katz, R.A., Maniatis, G.M., Guntaka, R.V., Translation of avian sarcoma virus RNA in Xenopus laevis oocytes. Biochem. Biophys. Res. Comm. 86, 447-453 (1979).

78. Gedamu, L., Dixon, G.H. and Gurdon, J.B., Studies on the injection of poly(A)+ protamine mRNA in Xenopus laevis oocytes, Exptl. Cell. Res. 117, 325-334 (1978).

79. Gruber, M. cited in: Campbell P.N. and Blobel, G., The role of organelles in the chemical modification of the primary translation products of secretory proteins. FEBS Lett. 72, 215-226 (1976).

80. Mous, J., Peeters, B., Rombauts, W. and Heyns, W. Synthesis of rat prostratic binding protein in Xenopus oocytesm and in wheat germ. Biochim. Biophys. Res. Commun. 79, 1111-1116 (1977).

81. Yip, C.C., Hew, C.-L. and Hsu, H. Translation of messenger ribonucleic acid from isolated pancreatic islets and human insulinoma. Proc. Natl. Acad. Sci. USA. 72, 4777-4776 (1975).

82. Kortbeek-Jacobs, N. and van der Donk, H. Detection of specific antibody producing cells in the porcine colostrum by in ovo translation of their mRNA. J. Immunol. Meth. 24, 195-199 (1978).

83. Palmiter, R.D., Gagnon, J., Vogt, V.M., Ripley, S. and Eisenman, R.N., The $NH_2$-terminal sequence of the avian oncovirus gag precursor polyprotein ($Pr76^{gag}$) Virology 91, 423-433 (1978).

Eur J Biochem 91 65–72 (1978)

IV - 2

# Post-translational Assembly of Lens α-Crystallin in the Reticulocyte Lysate and in *Xenopus laevis* Oocytes

Fred A M ASSELBERGS Marion KOOPMANS Walther J VAN VENROOIJ and Hans BLOEMENDAL

Department of Biochemistry University of Nijmegen

(Received May 19 1978)

Lens mRNA was translated in a reticulocyte lysate predominantly into monomeric α-crystallin chains Lens polyribosomes added to the cell free system produced the same polypeptides, but these were detected predominantly in α-crystallin aggregates Lens mRNA, after microinjection into *Xenopus laevis* oocytes, produced α-crystallin subunits that were exclusively found in the form of high-molecular weight complexes Also after injection of the purified 14-S mRNA, coding for the αA subunit the synthesized αA polypeptides were incorporated into high-molecular-weight aggregates In contrast, the synthesis of αB subunits, directed by a 10-S mRNA, did not result in aggregate formation The experiments thus suggest that aggregate formation of α-crystallin is triggered by its αA subunits, which are then joined by the αB subunits This process occurs partly in the cell free system and completely in *Xenopus* oocytes

For model studies on the assembly of a multimeric protein, calf lens α-crystallin shows very attractive features The native protein is a large aggregate, with an average molecular weight of 800000 composed of subunits of $M_r$ 20000 [1 2] There are two kinds of subunits, designated αA (A = acidic) and αB (B – basic), occurring in a ratio of about 2 1 [3] Synthesis of αA-crystallin is directed by a 14-S mRNA [4], while αB is coded for by a 10-S mRNA [4–7] In calf lenses about one third of the primary translation products ($\alpha A_2$ and $\alpha B_2$) is transformed into the deamidated chains $\alpha A_1$ and $\alpha B_1$ α-Crystallin is subject to ageing processes during which the molecular weight of the aggregate increases, while part of the polypeptide chains undergo C-terminal degradation [1,2]

In addition to α crystallin two other classes of proteins exist in the eye lens, namely β and γ-crystallin β-Crystallins occur in two size classes, named $\beta_H$ (high) and $\beta_L$ (low) and are composed of a number of subunits, some of which are shared by both β subclasses [8] γ-Crystallins are monomeric proteins with molecular weights of about 20000 The present knowledge of mammalian lens proteins and their biosynthesis has recently been reviewed by Harding and Dilley [1] and by Bloemendal [2]

In previous work from our laboratory, synthesis of α crystallin aggregates was studied in intact cultured lenses and in the lens cell-free system [9,10] In these studies, newly synthesized α-crystallin polypeptides were found only in high-molecular-weight aggregates and no monomeric or oligomeric α-crystallin was found However, the possibility that in these homologous translation systems newly synthesized α-crystallin polypeptide chains were incorporated into pre-existing α-crystallin aggregates was not excluded In another study a cell-free system from rabbit reticulocytes was programmed with lens polyribosomes [11] The translation products were then mixed with carrier crystallins and analyzed by gel filtration Again exclusively high-molecular-weight α-crystallin aggregates were found, but also in this case incorporation into pre existing aggregates may have occurred Therefore, it was decided in this study to analyze the products from heterologous translation systems in the absence of any carrier lens protein As cell-free system we chose one of the most efficient systems available at present namely the unfractionated reticulocyte lysate [12], rendered mRNA-dependent with micrococcal nuclease according to Pelham and Jackson [13]

Cell-free systems translate exogenous mRNA only for a short period and sometimes fail to assemble proteins, like for example uteroglobin [14] Therefore, we injected mRNA also in *Xenopus laevis* oocytes, which are capable of translating exogenous mRNA for several days [15] In this system assembly of proteins, namely uteroglobin [14] and immunoglobulin [16] has been reported With α-crystallin we found that in the reticulocyte lysate assembly occurred only partially, but that in living oocytes high-molecular-weight α-crystallin aggregates were formed exclusively In order to test which of the α-crystallin subunits was, under these conditions, the 'promoter' of aggregate formation, 10-S and 14-S mRNA were separately injected into oocytes The results indicate that newly

synthesized αA subunits but not αB subunits promote aggregation

## EXPERIMENTAL PROCEDURE

### *Materials*

L-[$^{35}$S]Methionine (spec act 400 500 Ci mmol) $^{14}$C-labeled protein hydrolysate (spec act 56 Ci mol) and a $^{3}$H labeled l amino acid mix of identical composition were purchased from the Radiochemical Centre (Amersham England) Creatine kinase was from Boehringer Mannheim and micrococcal nuclease (spec act $9\,7 \times 10^{3}$ units/mg) from P L Biochemicals Milwaukee

### *Isolation of Polyribosomes and mRNAs*

Calf lens polyribosomes were isolated following procedures described previously [17 18] From these polyribosomes total poly(A) containing mRNA was isolated by affinity chromatography on oligo(dT)-cellulose as described previously [19 20] The mRNA was separated into a 10 S and 14 S fraction by two subsequent centrifugations in isokinetic sucrose gradients [20] Translation in a reticulocyte cell free system showed that they were not cross contaminated with one another

### *Translation of mRNA*

Injection of mRNA or polyribosomes into *Xenopus* oocytes was performed essentially according to Gurdon et al [21 22] and has been described previously [19] 25 nl containing 100 ng polyribosomes or 5 ng lens mRNA was injected Oocytes were incubated for 18 h in modified Barth s medium containing either 0 5 mCi/ml L [$^{35}$S]methionine or $^{3}$H labeled L-amino acid mix or $^{14}$C labeled protein hydrolysate After incubation oocytes were frozen at $-20$ C Translation of lens mRNA in a nuclease-pretreated reticulocyte lysate has been described before [23]

### *Gel Filtration of Translation Products*

Portions of 5–10 oocytes were homogenized in 2 ml of gel filtration buffer (0 1 M ammonium formate, pH 7 3 0 1 mM EDTA) containing extra EDTA (5 mM) and glycerol (10%) The reticulocyte lysate incubations were dissolved in the same buffer The lysate was centrifuged for 10 min at $64000 \times g$ in a Spinco Ti 50 rotor The supernatant was then loaded onto a column of Sephadex G 200 (Pharmacia) ($125 \times 2$ cm) Fractions of 3 ml were collected Radioactivity was determined in a Packard liquid scintillation counter after mixing 1 ml samples with 10 ml of Aquasol (New England Nuclear) Molecular weights were estimated using cytochrome c ($M_r = 12500$) soybean trypsin inhibitor ($M_r = 20000$) ovalbumin ($M_r = 45000$) bovine serum albumin ($M_r = 68000$) and rabbit immunoglobulin G ($M_r - 150000$) as markers Pooled fractions were lyophilized and processed for immunoprecipitation or electrophoretic analysis [11]

### *Immunoprecipitation of Lens Proteins*

Lyophilized column fractions were dissolved in 1 ml NaCl $P_i$/dodecylsulphate (10 mM sodium phosphate pH 7 2 0 9% NaCl 0 3% Triton-X-100 0 5%, sodium deoxycholate and 0 1% sodium dodecylsulphate) Some undissolved material was removed by centrifugation at $200000 \times g$ in a Ti 50 rotor for 10 min Conditions for direct immunoprecipitation of lens proteins have been described [19] Immunoprecipitates were collected and dissolved in the sample buffer for analysis by dodecylsulphate polyacrylamide gel electrophoresis as described by Van Zaane et al [24]

### *Reaggregation of α Crystallin*

Translation products from oocytes were prepared for gel filtration as described above To an equivalent of five oocytes lens crystallins (final concentration 0 1 mg ml) and urea (final concentration 6 M) were added in order to dissociate crystallin aggregates After incubation for 1 h at 0 C urea was removed by gel filtration on Sephadex G 25 (coarse) allowing reaggregation to occur [25]

## RESULTS

### *Are α-Crystallin Aggregates Formed in the Reticulocyte Lysate?*

To study the size distribution of lens crystallins formed in the reticulocyte lysate translation products labeled with [$^{35}$S]methionine were subjected to gel filtration on Sephadex G-200 (Fig 1) α-Crystallin isolated from calf lenses elutes with the void volume followed by $\beta_H$ $\beta_L$ and $\gamma$ crystallin respectively When the translation products of purified poly(A) containing lens mRNA were analyzed by gel filtration no labeled products were found in the void volume fraction (Fig 1B) although there was some material that eluted just behind it (molecular weight about 250000) Most of the products eluted at the position where $\beta_L$ and $\gamma$-crystallin are expected These products had apparent molecular weights of 40000 and 16000 respectively The polypeptides made under the direction of lens polyribosomes were found in much larger aggregates some of them eluting with the void volume (Fig 1A) There was also more material eluting in the $\beta_L$ range ($M_r$ 30000 50000) When a

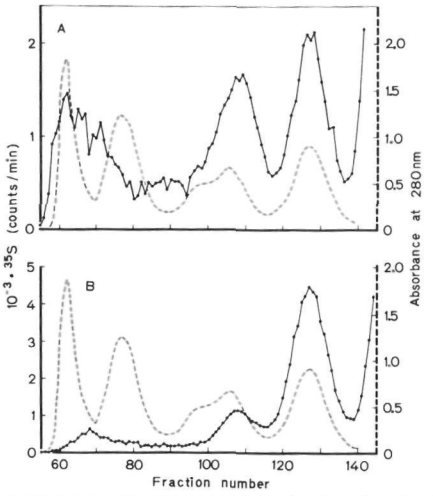



Fig. 1. *Gel filtration of lens protein synthesized in the nuclease-treated reticulocyte lysate.* Lens polyribosomes were translated in an mRNA-dependent reticulocyte lysate as described under Experimental Procedures. Translation products were separated by gel filtration on Sephadex G-200 without the use of carrier proteins (A). In a separate run soluble lens proteins were resolved into α, $\beta_H$, $\beta_L$ and γ-crystallin fractions (——). Unincorporated [$^{35}$S]methionine elutes in fractions 140–155. (B) Elution profile of products synthesized in a reticulocyte lysate under the direction of total poly(A)-containing lens mRNA

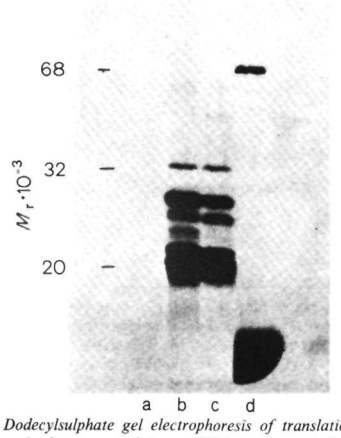



Fig. 2. *Dodecylsulphate gel electrophoresis of translation products of lens polyribosomes and lens mRNA in the reticulocyte lysate.* Total lens mRNA (b) and lens polyribosomes (c) were translated in the mRNA-dependent reticulocyte cell-free system and translation products were separated in a dodecylsulphate-containing polyacrylamide gel autoradiographed as described under Experimental Procedure. A control incubation without mRNA is shown in slot (a), endogenous proteins synthesized in a reticulocyte lysate without treatment with micrococcal nuclease in slot (d). The positions of two lens proteins, αA ($M_r$ 20000) and $\beta B_{1a}$ ($M_r$ 32000), are shown together with the position of globin and of a 68000-$M_r$ polypeptide synthesized by rabbit reticulocytes

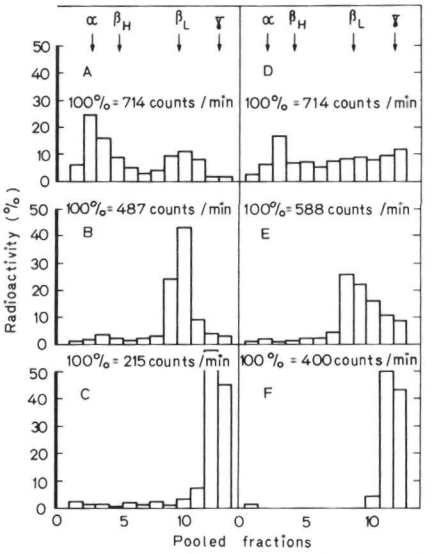



Fig. 3. *Immunoprecipitation of lens proteins from the Sephadex G-200 fractions of the reticulocyte lysate.* Translation products of lens polyribosomes (A–C) and lens mRNA (D–F) were separated by gel filtration (see Fig. 1). Fractions were pooled, then lyophilized and immunoprecipitation was subsequently carried out with antisera to αA-crystallin (A and D), total β-crystallin (B and E) and γ-crystallin (C and F), respectively. The positions at which native crystallins eluted in a separate run are indicated by arrows

control incubation (no mRNA added) was chromatographed no material eluted before fraction 140 (not shown). The finding that only after the addition of lens polyribosomes to the cell-free system were high-molecular-weight crystallin aggregates formed was unexpected since the individual polypeptides synthesized under the direction of lens mRNA were almost identical with those directed by the lens polyribosomes (Fig. 2).

In order to identify more precisely the subunit composition of the synthesized protein aggregates, pooled gel filtration fractions were immunoprecipitated with anti-crystallin antisera (Fig. 3).

The distribution of the αA-crystallin subunits showed that translation of lens polyribosomes produced somewhat more α-crystallin aggregates and of higher molecular weight than did mRNA. In the first three fractions 46% and 28% of all immunoprecipitable αA-crystallin was eluted respectively (Fig. 3 A, D). Immunoprecipitation with antisera to β and γ-crystallin (Fig. 3 B, C, E, F) confirmed that, as expected, they were not eluting with the void volume. β-Crystallin polypeptides were found as monomers or small aggregates with the size of $\beta_L$. γ-Crystallin polypeptides did not form aggregates.

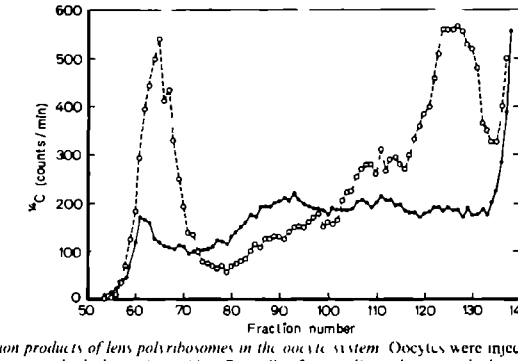



Fig 4 *Gel filtration of translation products of lens polyribosomes in the oocyte system* Oocytes were injected with 0 1 µg lens polyribosomes and labeled with $^{14}$C containing protein hydrolysate (O O) α Crystallin from calf eye lenses with the void volume (fraction 60 63) For comparison the elution profile of $^{14}$C -labeled oocytes injected with 25 nl water is shown (● - ●)

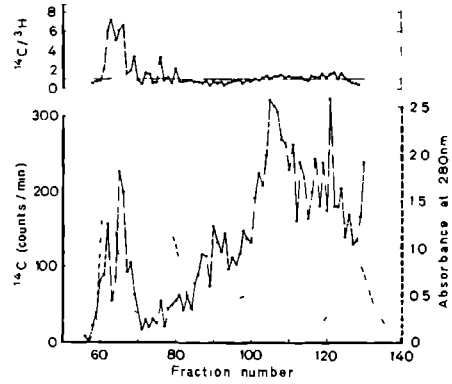



Fig 5 *Gel filtration of translation products of lens mRNA in oocytes* Oocytes were injected with 5 ng lens mRNA and labeled with $^{14}$C-containing protein hydrolysate for 20 h Water injected oocytes were labeled with $^{3}$H containing amino acids Five oocytes of each incubation were mixed homogenized and the homogenate was subjected to gel filtration Only the $^{14}$C elution profile is shown (● —●) together with the $^{14}$C $^{3}$H ratio (upper part) Because the inputs of $^{3}$H and $^{14}$C were different the $^{14}$C $^{3}$H ratio was normalized to 1 The elution profile of unlabeled lens proteins was determined in a separate run ( )

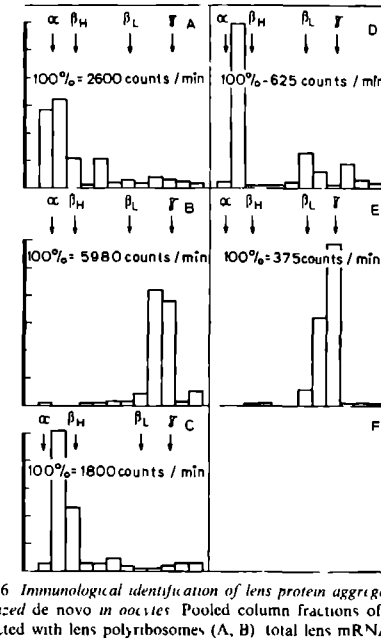



Fig 6 *Immunological identification of lens protein aggregates synthesized de novo in oocytes* Pooled column fractions of oocytes injected with lens polyribosomes (A, B) total lens mRNA (D, E) or 14 S mRNA (C) were first lyophilized and then immunoprecipitated with antiserum to αA-crystallin (A D C) and γ-crystallin (B E) The positions of the crystallins were determined for each column in a separate run and are indicated by arrows

### α-Crystallin Formation in Oocytes

Oocytes were injected with water or 0 1 µg of lens polyribosomes and were labeled for 18 h with $^{14}$C-labeled protein hydrolysate The translation products were analyzed by gel filtration (Fig 4) Two peaks of labeled lens proteins were eluted one just behind the void volume and another at the position of γ-crystallin with a shoulder in the $\beta_L$ region In order to correct for the endogenous protein synthesis of the oocyte a double-label experiment was performed Oocytes were injected with lens mRNA and labeled with $^{14}$C-labeled protein hydrolysate, at the same time water-injected oocytes were labeled with $^{3}$H-labeled protein hydrolysate After incubation both batches of oocytes were mixed and analyzed together (Fig 5) The very high $^{14}$C $^{3}$H ratio in the peak near the void volume indicates that most of that material consisted of translation products of lens mRNA Although radioactivity in the $\beta_L$ and γ-crystallin region varied between oocytes from different frogs, the $^{14}$C/$^{3}$H ratio

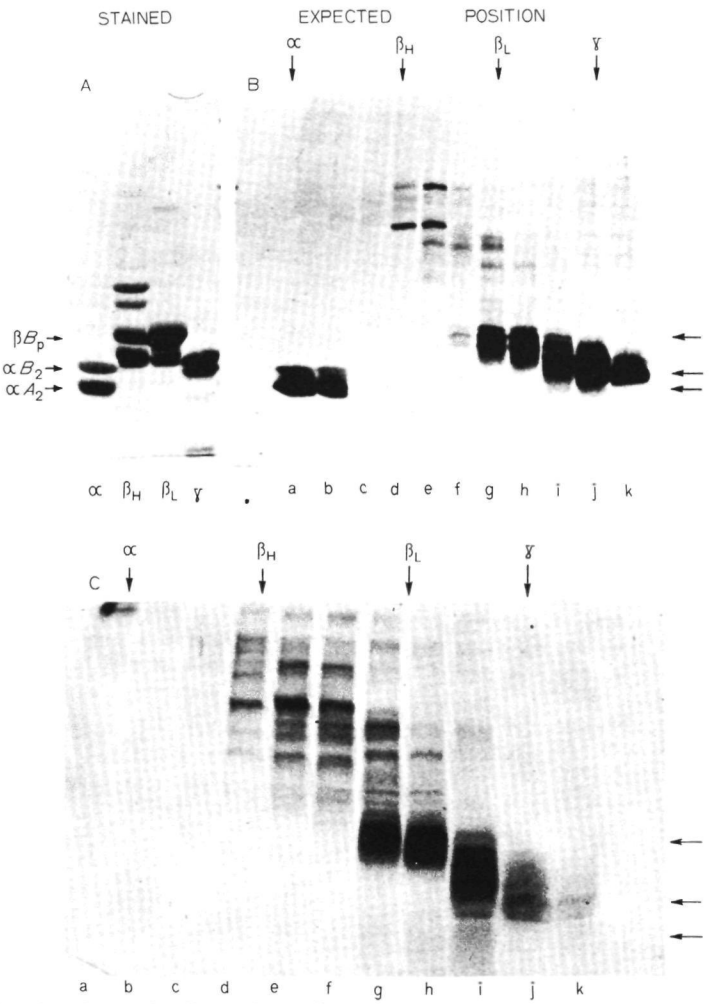



Fig. 7. *Dodecylsulphate electrophoresis of gel filtration fractions from oocytes programmed with total lens mRNA or the 10-S fraction of lens mRNA.* After gel filtration pooled fractions were lyophilized and analyzed on dodecylsulphate gels as described under Experimental Procedure. As indicated above the figures, native α-crystallin is expected to elute in fraction a, $\beta_H$ in d, $\beta_L$ in g and h, and γ-crystallin in fraction j and k. The translation products of total lens mRNA (B) and the 10-S fraction (C) are shown. For comparison the stained gel of purified lens proteins is shown in (A) and the positions of α-crystallin subunits, αA and αB, as well as the main subunit of β-crystallin aggregates, βBp, are indicated

always indicated the presence of some newly synthesized lens proteins in the 20000–40000-$M_r$ region. A similar double-label experiment with oocytes injected with lens polyribosomes gave identical results (not shown).

An immunoprecipitation procedure with antiserum to αA and γ-crystallin was used to identify the high-molecular-weight protein aggregate isolated by gel filtration (Fig. 6). Most of the αA-crystallin was found to be present in the first peak eluting from the column, regardless of whether lens polyribosomes or purified mRNA had been injected (Fig. 6A, D). There was no γ-crystallin present in the void volume (Fig. 6B, E), indicating that no aspecific aggregation had taken place. Direct analysis of pooled fractions on a dodecylsulphate-containing polyacrylamide gel confirmed the presence of αA and αB subunits in the high-molecular-weight aggregate (Fig. 7B).

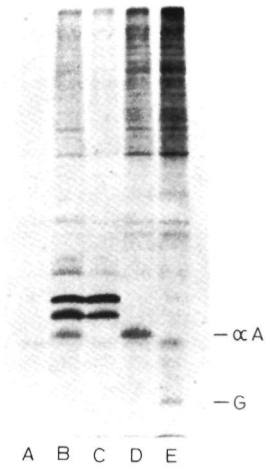



Fig. 8. *Dodecylsulphate gel electrophoresis of translation products of lens mRNA in oocytes.* Oocyte homogenates labeled with [$^{35}$S]methionine were directly analyzed on dodecylsulphate gels: (A) oocytes injected with 25 nl water; (B) injected with total lens mRNA; (C) with 10-S mRNA; (D) with 14-S fraction of lens mRNA; (E) injected with 9-S mRNA from rabbit reticulocytes. The position of calf αA-crystallin and of rabbit globin (G) is indicated

### *Aggregation of Newly Synthesized αA Subunits after Translation of 14-S mRNA in Oocytes*

Purified αA polypeptide chains can form high-molecular-weight aggregates after dissociation and reassociation with urea [26–28]. Therefore, it seemed interesting to see if translation of 14-S mRNA into αA-crystallin produced an aggregate. Analysis of 14-S translation products confirmed the sole presence of αA-crystallin (Fig. 8D). However, after gel filtration and immunoprecipitation virtually no monomeric αA chains could be detected (Fig. 6C). Most of the αA was found in aggregates, eluting just behind the void volume, with estimated molecular weights of 200000–400000.

### *Are αB Subunits Incorporated into α-Crystallin Aggregates?*

In order to verify if αB chains alone were also able to form a polymeric protein, 10-S mRNA was translated in oocytes. This mRNA fraction contains also messengers for β and γ-crystallin polypeptides, but is free of mRNA coding for αA, as can be seen after gel electrophoresis of oocytes injected with the 10-S mRNA (Fig. 8C). In a double-label experiment of the type described above, no large translation products were detected (not shown) as was the case with 14-S mRNA products (Fig. 6C). However, there was a considerable amount of newly synthesized material in the $M_r$ range of 20000–40000. The translation products were separated by gel filtration and then analyzed by dodecylsulphate/polyacrylamide gel electrophoresis (Fig. 7C). The results confirmed the absence of αB aggregates. In contrast, when total lens mRNA (this is 10-S plus 14-S mRNA) was injected, both αA and αB chains were found in aggregates eluting just behind the void volume (Fig. 7B).

In order to verify whether any potentially functional αB-crystallin was formed in unaggregated form in oocytes after injection with 10-S mRNA, a dissociation-reaggregation experiment was carried out. It is known that α-crystallin aggregates dissociate in urea or guanidine-HCl and reaggregate, when the dissociating agent is removed. To homogenates of oocytes injected with 10-S mRNA, urea (final concentration 6 M) and in some cases carrier crystallins were added. After incubation for an hour at 0–4 °C the urea was removed by gel filtration on Sephadex G-25. During removal of the urea, specific reaggregation of the dissociated α-crystallin polypeptides occurs, provided the experiment is performed at a low crystallin concentration [25]. Our results showed that only in the presence of carrier crystallins did coaggregation of radioactive αB polypeptides occur (Fig. 9B). Subsequent analysis of the void volume fraction by dodecylsulphate/polyacrylamide gel electrophoresis showed that the increase in radioactivity was due to a polypeptide of $M_r$ about 22000 (not shown). This polypeptide can not be γ-crystallin, since γ-crystallin does not reaggregate under these conditions [25]. The results therefore suggest that in oocytes injected with 10-S mRNA potentially functional αB polypeptides, capable of being incorporated into α-crystallin aggregates, were formed.

## DISCUSSION

In the intact lens 40 or more α-crystallin subunits ($\alpha A_2$ and $\alpha B_2$) combine to form the mature α-crystallin aggregate. Previous studies in the lens cell-free system showed that complete aggregation of newly synthesized αA and αB chains occurs [10]. However, the possibility that these aggregates were not completely synthesized *de novo* was not excluded, since this system already contains a large amount of α-crystallin before synthesis of radioactive subunits starts. Both α-crystallin subunits can also be synthesized in a reticulocyte lysate. Vermorken et al. [11] studied crystallin aggregate formation in the reticulocyte lysate, but after translation of lens polyribosomes they added lens proteins as carrier before gel filtration. Thus, also from their study it can not be concluded that complete

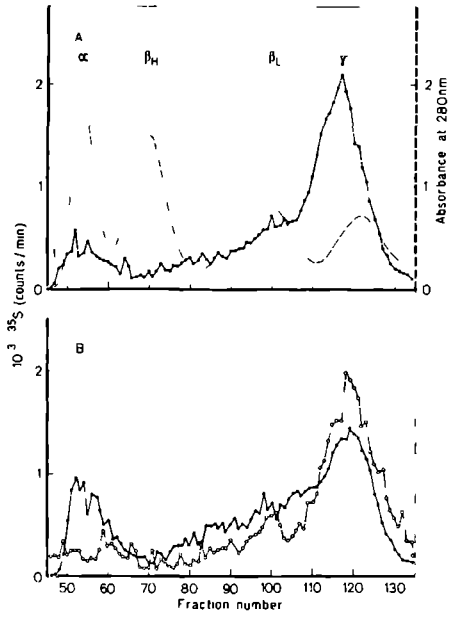



Fig 9 *Reaggregation of oocyte derived αB crystallin into high molecular weight α crystallin* Oocytes were injected with 5 ng 10 S mRNA and labeled for 18 h with [$^{35}$S]methionine (A) Translation products were analyzed by gel filtration as described in Methods (● —●) The position of the crystallin markers was determined in a separate run ( ) (B) Translation products were dissociated in 6 M urea in the absence (O O) and presence (● ●) of 0 1 mg ml$^{-1}$ α crystallin After removal of the urea the reassociated translation products were separated on Sephadex G 200 columns as described above

*de novo* formation of α-crystallin takes place Indeed, when we separated translation products of lens mRNA in the reticulocyte lysate in the absence of carrier lens crystallins a considerable amount of monomeric αA-crystallin was found (Fig 1 and 3) next to small α-crystallin aggregates of molecular weights ranging from 40000 to 200000 Presumably aggregation was not complete, because the amount of α-crystallin polypeptides synthesized was not sufficient to allow aggregation in the absence of carrier α-crystallin

When lens polyribosomes were translated in the cell-free system, not only more aggregate formation was found, but also some fully sized α-crystallin aggregates ($M_r$ greater than 400000) had been formed The efficiency of translation per mRNA molecule was virtually equal when lens polyribosomes or purified mRNA were added to the cell-free system (Asselbergs, unpublished) Therefore, the most plausible explanation is that these aggregates were formed by combination of newly synthesized α crystallin subunits with pre-existing α-crystallin present on the polyribosomes added to the reticulocyte lysate Some α crystallin remains associated with the polyribosomes even after treatment with 0 5 M KCl or 0 5% desoxycholate (Asselbergs unpublished) and these α crystallin molecules might represent partial aggregates Apparently when the concentration of α-crystallin polypeptides is sufficiently high aggregation into high molecular-weight complexes occurs very fast When carrier α-crystallin is present this aggregation occurs instantaneously and this explains the results of previous experimentators [9–11]

*Xenopus* oocytes injected with lens mRNA, synthesized high molecular-weight α-crystallin in the absence of carrier protein (Fig 5 and 6) Presumably this was because oocytes have two advantages over a cell-free system in synthesizing multimeric proteins Firstly they produce more of the translation product than a cell-free system does and synthesis proceeds for a longer period For example it has been reported that in the case of interferon mRNA this can result in the synthesis of 500 times more translation product in oocytes than in a cell-free system [30] Secondly, oocytes reinitiate mRNA translation more frequently If aggregation is started by the nascent polypeptides still attached to the polyribosomes as has been suggested for collagen [31] it is necessary to have a translation system capable of frequent reinitiation of protein synthesis

Surprisingly, αA subunits alone synthesized in oocytes under the direction of 14-S mRNA could also form aggregates (Fig 6C) while αB subunits coded for by a 10-S mRNA did not form high molecular-weight complexes in the absence of the αA subunit (Fig 7C) After addition of carrier α crystallin the radioactive αB subunits were incorporated into high-molecular-weight α-crystallin during a dissociation reassociation experiment (Fig 9) From these observations we conclude that, at low concentrations of α crystallin, subunit aggregate formation is triggered by the αA subunits, which subsequently are joined by the αB subunits Our results indicate that these processes occur partially in a cell-free system and almost completely in living *Xenopus* oocytes

The present investigations have partly been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid of the Netherlands Organization for the Advancement of Pure Research (ZWO)

## REFERENCES

1 Harding J J & Dilley H J (1976) *Exp Eye Res 22* 1–73
2 Bloemendal H (1977) *Science (Wash D C) 197* 127–137
3 Van Kamp G J Schats I H M & Hoenders H J (1973) *Biochim Biophys Acta 295* 149–151
4 Berns A J M van Kraaikamp M Bloemendal H & Lane C D (1972) *Proc Natl Acad Sci U S A 69* 1606–1609

5 Berns, A J M , Strous, G J A M & Bloemendal, H (1972) *Nat New Biol 236*, 7–9
6 Matthews, M B , Osborn, M , Berns, A J M & Bloemendal, H (1972) *Nat New Biol 236*, 5–7
7 Berns A J M , Schreurs, V V A M , van Kraaikamp, M W G & Bloemendal, H (1972) *Eur J Biochem 33*, 551–557
8 Herbrink, P van Westreenen, H & Bloemendal, H (1975) *Exp Eye Res 20*, 541–548
9 Strous, G J A M , van Westreenen, H & Bloemendal, H (1973) *Eur J Biochem 38*, 79–85
10 Strous G J A M , van Westreenen, H , van der Logt J & Bloemendal, H (1974) *Biochim Biophys Acta, 353*, 89–98
11 Vermorken, F J M , Herbrink, P & Bloemendal, H (1977) *Eur J Biochem 78*, 617–622
12 Adamson, S D , Howard, G A & Herbert, F (1969) *Cold Spring Harbor Symp Quant Biol 34*, 547 -554
13 Pelham, H R B & Jackson, R J (1976) *Eur J Biochem 67*, 247–256
14 Beato, M & Nieto, A (1976) *Eur J Biochem 64* 15 25
15 Gurdon, J B , Lingrel, J B & Marbaix, G (1973) *J Mol Biol 80* 539–551
16 Stevens, R & Williamson, A R (1973) *J Mol Biol 78*, 517–525
17 Bloemendal, H Schoenmakers, J J G , Zweers, A , Matze, R & Benedetti, E L (1966) *Biochim Biophys Acta, 123*, 217 220
18 Berns A J M & Bloemendal, H (1974) *Methods Enzymol 30*, 675–694
19 Asselbergs, F A M , van Venrooij, W J & Bloemendal, H (1978) *Eur J Biochem 87*, 517–524
20 Cohen, L H , Smits, D P F M & Bloemendal H (1976) *Eur J Biochem 67*, 563–572
21 Lane, C D , Marbaix G & Gurdon, J B (1971) *J Mol Biol 61*, 73–91
22 Gurdon J B Lane C D , Woodland, H R & Marbaix, G (1971) *J Mol Biol 233*, 177–182
23 Asselbergs, F A M , Peters, W , van Venrooij W J & Bloemendal H (1978) *Biochim Biophys Acta 520* 577–587
24 Van Zaane D , Gielkens, A J I , Dekker Michielsen, M J A & Bloemers, H P J (1975) *Virology, 67* 544–552
25 Bloemendal H , Zweers, A , Benedetti L L & Walters, H (1975) *Exp Eye Res 20*, 463–478
26 Li L K & Spector, A (1972) *Exp Eye Res 13* 110–119
27 Spector A , Li L-K , Meretsky, D & Augusteyn, R (1971) *Am J Ophthalmol 71*, 386 390
28 Li, I -K & Spector, A (1973) *Exp Eye Res 15*, 179–183
29 Bloemendal H , Bont, W S , Jongkind J F & Wisse, J H (1962) *Exp Eye Res 1*, 300 305
30 Reynolds, F H , Jr, Premkumar, F & Pitha, P M (1975) *Proc Natl Acad Sci U S A 72*, 4881–4885
31 Veis, A & Brownell, A G (1977) *Proc Natl Acad Sci U S A 74*, 902–905

F A M Asselbergs M Koopmans, W J van Venrooij, and H Bloemendal, Laboratorium voor Biochemie, Katholieke Universiteit Nijmegen, Geert Grooteplein Noord 21, NL-6525 EZ Nijmegen, The Netherlands

# Chapter V

## TRANSLATION OF VIRUS-SPECIFIC MESSENGER RNA IN XENOPUS OOCYTES

V-1 Introduction: Polyprotein cleavage and subgenomic mRNAs.

V-2 Translation of oncogenic virus RNA in Xenopus laevis oocytes.

V-3 Kinetics of synthesis and proteolytic processing of precursor polypeptides of Rauscher murine leukemia virus in frog oocytes following microinjection of viral RNA.

V-1

Introduction: Polyprotein cleavage and subgenomic mRNAs

Virologists have used the oocyte system primarily because of its capacity for post-synthetic modification of the viral translation products. This subject has already been reviewed in section IV-1. This chapter deals with a study of the post-synthetic cleavage of precursor polypeptides, "polyproteins", of two retrovirus species, avian myeloblastosis virus (V-2) and Rauscher murine leukemia virus. (V-3) Oocytes have two important advantages for the study of retrovirus polyprotein processing. Firstly, the translation of the viral RNA occurs in a cell, which up till the moment of injection does not contain any product of viral gene expression. Secondly, the processing of the viral precursor polypeptides occurs with a much lower rate than in the normal host cell of the virus (Ghysdael et al., 1977), which facilitates the study of the cleavage mechanism.

Another feature of the translation of viral RNA in oocytes is the recognition of the proper initiation region of those viral RNAs, which are, in a genetic sense, polycistronic (for a review see: Kozak, 1978). Such RNAs, usually isolated from virus particles (genomic RNAs), contain the genetic information for the synthesis of two or more polypeptides, but in general only the genetic information proximal to the 5'-terminus of the RNA molecule is translated into protein <u>in vitro</u>. Only in a few cases it has been reported that the internal cistron was translated in a cell-free system (Rutgers, 1977; van Vloten-Doting et al., 1977; references in Kozak, 1978). The great advantage of the oocyte system, in this respect, is that the injected RNAs are translated in a "normal"

intracellular environment as opposed to the "artificial" environment in a cell-free system. Therefore, the use of oocytes as a translation system may eliminate certain in vitro artifacts in recognition of the initiation sites on the mRNA by the ribosomes. In addition of genome-size RNAs smaller "subgenomic" mRNAs are found in virus-infected cells. These mRNAs, which contain primarily nucleotide sequences from the 3' part of the genomic RNAs, can be translated in vitro into the other virus-encoded polypeptides. Thus via subgenomic mRNAs the virus is able to express the genetic information that is silently present in the genomic (or genome-size) mRNAs.

Several polycistronic viral RNAs have been injected into Xenopus oocytes, notable the genomic RNAs of four plant viruses and the genomic RNAs of several retroviruses (see: section II, table 4). In the case of the plant viruses the information encoding the coat protein is silently present in the genomic RNA (or in one of the genome RNAs of viruses with multipartite genomes). The genomic RNA of the retroviruses contains the silent cistron for the proteins of the viral envelope. However, in none of these cases expression of the silent cistrons could be detected in oocytes injected with genomic RNA, although the polypeptides were synthesized after injection of the subgenomic mRNAs. (After injection of alfalfa mosaic virus genome RNA 3 synthesis of the coat protein was observed in oocytes (Rutgers et al., 1976; Van Vloten-Doting et al., 1977), but this observation could not be repeated when more rigorously purified RNA 3 preparations were injected (Rutgers, 1977)). Also in the case of the two retroviruses studied by us, avian myeloblastosis virus (V-2) and Rauscher murine leukemia virus (V-3) no

expression of the internal cistron was observed when genomic-size RNA was injected. In our opinion these findings also imply that the injected genomic RNAs are also not converted to subgenomic mRNAs by enzymes present in the oocytes.

References

1. Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D.P., Burny, A., Cleuter, Y., Huez, G., Kettman, R., Marbaix, G., Portetelle, D. and Chantrenne, H., (1977) Frog oocytes synthesize and completely process the precursor polypeptide to virion structural proteins after microinjection of avian myeloblastosis virus. Proc. Natl. Acad. Sci. USA 74, 3230-3234.

2. Kozak, M. (1978) How do eukaryotic ribosomes select initiation regions in messenger RNA? Cell 15, 1109-1123.

3. Rutgers, A.S., (1977) In vivo and in vitro translation of the RNAs of alfalfa mosaic virus. Thesis, State University of Leiden, Leiden, The Netherlands.

4. Rutgers, T., Neeleman, L., van Vloten-Doting, L., Cleuter, Y., Hubert, E., Huez, G., and Marbaix, G. (1976) Translation products of the alfalfa mosaic virus ribonucleic acids in wheat germ cell-free system and in oocytes from Xenopus laevis. Arch. Int. Physiol. Biochem. 84, 654-655.

5. Van Vloten-Doting, L., Bol. J., Neeleman, L., Rutgers, T., van Dalen, D., Castel, A., Bosch, L., Marbaix, G., Huez, G., Hubert, E. and Cleuter, Y., (1977) In vivo and in vitro translation of the RNAs of alfalfa mosaic virus. in: Nucleic acid and protein synthesis in plants (Borograd, L. and Weil, J.H., eds), pp. 387-411, Plenum Press, New York.

(Reprinted from Nature, Vol. 259, No. 5545, pp. 696–699, February 26, 1976)

# Translation of oncogenic virus RNA in *Xenopus laevis* oocytes

OOCYTE injection as developed by Gurdon *et al.*[1–3] is efficient and reliable for the assay of faithful translation of heterologous messenger RNA[2–6]. But, working in collaboration with Gurdon[7], we found that oncogenic viral RNA was not translated to a detectable extent in oocytes. Only in a cell-free system derived from *Escherichia coli* could we translate viral RNA into distinct polypeptides in the molecular weight range of the viral group-specific proteins[8]. Recently we achieved translation of Rauscher leukaemia virus (RLV) RNA and avian myeloblastosis virus (AMV) RNA in various cell-free systems, using immunoprecipitation for the analysis[9,10]. This verified that our failure to find virus-specific polypeptides in the oocyte system programmed with oncogenic viral RNA was due to the inadequacy of the methods available to detect translation when it occurs at low efficiency. We now report the synthesis of AMV-specific precursors and structural proteins in the oocyte system, detected by specific immunoprecipitation and scintillation autoradiography.

AMV contains single stranded RNA encapsulated by several proteins and a lipid envelope. The four major proteins, the so-called group-specific (gs) antigens immunologically indistinguishable among all avian oncornaviruses[11], are within the viral envelope[12].

There are additional glycosylated proteins, carrying the type-specific (ts) antigens, on the surface of the virion[13]. It is not known how many proteins are encoded by the viral RNA and how many cellular proteins become encapsulated in the virions during budding. To determine virus-specific proteins it is important to characterise the coding capacity of the viral RNA in conditions closely related to the *in vivo* situation.

The results of a typical translation experiment of AMV RNA are shown in Fig. 1. Because of the relatively high rate of endogenous protein synthesis of this system, immunoprecipitation with specific antisera is a prerequisite for detection of the viral proteins. Injection of native 60S AMV RNA results in synthesis of virus-specific polypeptides with molecular weights of 76,000, 66,000, 32,000, 25,000, 18,000 and 12,000. In contrast, denatured 35S AMV RNA leads to the synthesis of a small quantity of the precursor polypeptide with molecular weight 76,000. In chick embryo fibroblasts (CEF) infected with AMV the same precursors and structural polypeptides are found as have been synthesised after injection of oocytes with native 60S AMV RNA (compare Fig. 1*b* and *d*).

In addition to the five precursor polypeptides already described[14] we found in AMV-infected CEF a sixth unstable precursor of molecular weight 85,000 not present in uninfected CEF (Fig. 2). This pr85 was, however, not detectable after injection of AMV RNA into oocytes. Incubation of native 60S AMV RNA in a reticulocyte cell-free system (Fig. 3) resulted in the synthesis of the same precursors and structural polypeptides found in the oocyte system, but in different proportions (compare Figs 1 and 3). In this cell-free system pr76 is the predominant product and almost no cleavage products are present. This probably means that the processing of AMV precursors in the oocyte and in infected CEF is almost identical, whereas in the reticulocyte cell-free system almost no cleavage occurs. In other reticulocyte preparations processing of the 76,000-dalton precursor resembled the situation *in vivo* (Fig. 4). Whereas

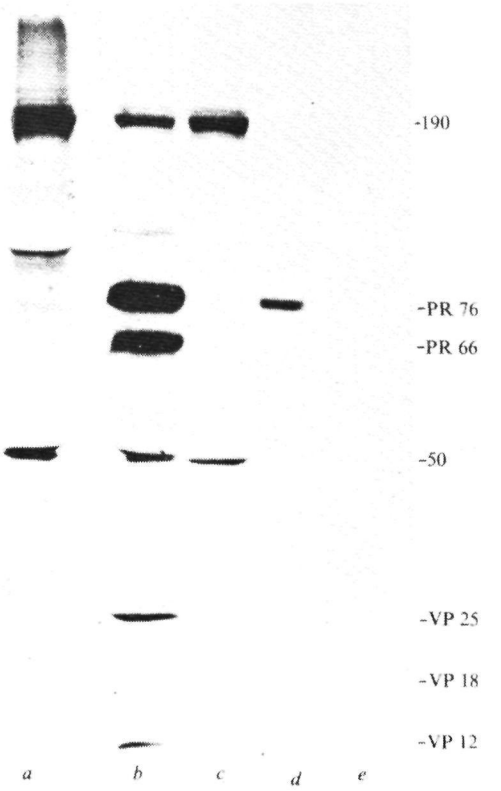



**Fig. 1** Autoradiography of an SDS gel electrophoresis pattern of immunoprecipitates. *Xenopus laevis* oocytes were injected by the method of message-injection described by Gurdon *et al.*[1]. AMV was obtained from viraemic plasma[15] and treated with SDS and Pronase. The 60S component of AMV RNA was isolated by centrifugation in isokinetic glycerol gradients[8]. Isolated 60S AMV RNA was denatured by heating at 100 °C for 3 min. The AMV-specific polypeptides synthesised in the oocytes in the presence of 2 μCi $^{35}$S-methionine (specific activity 478 mCi mmol$^{-1}$) per oocyte were detected by indirect immunoprecipitation[16] with an antiserum directed against the gs antigens of AMV. Primary cultures of $1 \times 10^6$ chick embryo fibroblasts infected with AMV were pulse labelled for 30 min with 50 μCi $^{35}$S-methionine (specific activity 210 Ci mmol$^{-1}$) in Earle's saline. After lysis in buffer indirect immunoprecipitation was carried out to detect virus-specific polypeptides. The immunoprecipitates were analysed on sodium dodecyl sulphate (SDS)–polyacrylamide slab gel gradients (7–18%) according to Berns *et al.*[17]. After staining and destaining, the slab gel was treated with DMSO–PPO[18], dried under vacuum and exposed in contact with a Kodak X-ray film for 3 d. *a*, Each of 20 oocytes was injected with 25 nl of sterilised distilled water; *b*, each of 10 oocytes was injected with 25 nl of native 60S AMV RNA (1 mg ml$^{-1}$) in distilled water; *c*, each of 10 oocytes was injected with 25 nl of heat-denatured 35S AMV RNA (1 mg ml$^{-1}$); *d*, immunoprecipitate from pulse-labelled chick embryo fibroblasts infected with AMV; *e*, immunoprecipitate from pulse-labelled uninfected chick embryo fibroblasts. PR, precursor polypeptide; VP, virion polypeptide. Numerals denote molecular weight in kdaltons.

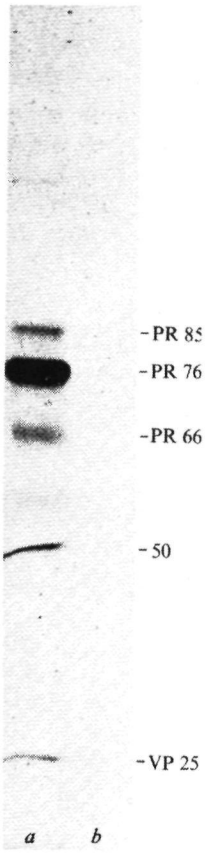



**Fig. 2** Autoradiograph of an SDS gel electrophoresis pattern of immunoprecipitates. Primary cultures of $1 \times 10^7$ chick embryo fibroblasts either uninfected or infected with AMV were pulse labelled for 30 min and immunoprecipitated as described in Fig. 1. The immunoprecipitates were analysed on slab gel gradients and exposed in contact with a Kodak X-ray film for 3 d. *a*, $1 \times 10^7$ infected cells precipitated with anti-AMV serum; *b*, $1 \times 10^7$ uninfected cells precipitated with anti-AMV serum.

the synthesis of the 76,000-dalton precursor polypeptide in a reticulocyte cell-free system is detectable even without immunoprecipitation (Fig. 3), as a rule immunoprecipitation procedures are necessary to visualise the small amount of virus-specific polypeptides. The specificity of the immunoprecipitation was checked as follows. Immunoprecipitation of control injections without mRNA (Fig. 1), by addition of anti-AMV serum, gives rise predominantly to two polypeptides with molecular weights of 190,000 and 50,000, respectively. The latter aspecific polypeptide is also always found when the reticulocyte lysate is the assay system. The same two polypeptides are detected as the only components after injection of AMV 60S RNA into the oocyte system followed by the addition of an antiserum directed against the non-related murine RLV (not shown).

From results obtained *in vivo* and *in vitro* we conclude that AMV RNA is translated into several precursor polypeptides which are cleaved into structural viral proteins, presumably by the same relatively unspecific enzymes present in chick embryo fibroblasts, oocytes and reticulocytes. We therefore believe that the oocyte system provides a useful tool for the study of the regulation processes involved in the expression of different viral functions *in vivo*.

We thank Dr John Gurdon for hospitality to M.S. We also thank Mrs Anne-Marie Selten-Versteegen for technical assistance, Dr J. W. Beard for viraemic chicken plasma and Dr G. de Boer for leukosis-free chick embryos. This work was supported in part by the Netherlands Foundation for Chemical Research and the Netherlands Organisation for Pure Research.

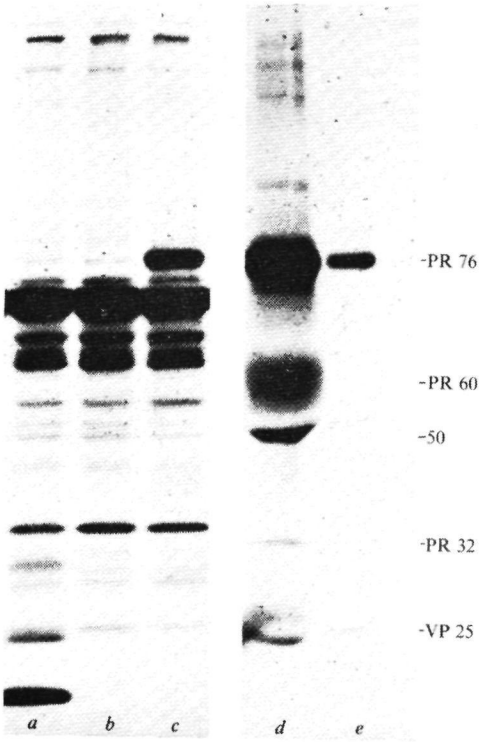



**Fig. 3** Autoradiograph of the SDS gel electrophoresis pattern of products synthesised in reticulocyte cell-free incubations. Native 60S AMV RNA was incubated for 60 min at 30 °C in a reticulocyte cell-free system as described elsewhere[10]. The final incubation volume was 25 µl. 2 µl of the incubation mixture was analysed directly on the SDS slab gel and another 20 µl was immunoprecipitated[16] and analysed. The Kodak X-rayfilm was exposed in contact with the dried gel for 2 d. *a*, Incubation in the reticulocyte cell-free system with lens 14S mRNA; *b*, control incubation without mRNA; *c*, incubation programmed with native 60S AMV-RNA; *d*, immunoprecipitate of an incubation with native 60S AMV RNA; *e* immunoprecipitate of polypeptides present in pulse labelled AMV-infected chick embryo fibroblasts.

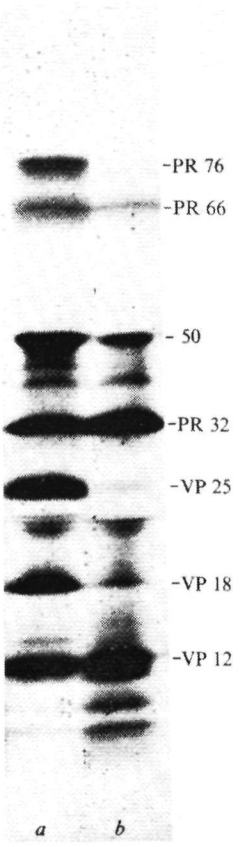



**Fig. 4** Autoradiography of immunoprecipitated products synthesised in the reticulocyte cell-free incubation and analysed by SDS gel electrophoresis. The analysis of the virus-specific polypeptides synthesised in the reticulocyte cell-free system were as described in the legend to Fig. 3. The X-ray film was exposed in contact with the dried gel for 3 d. *a*, Immunoprecipitation of an incubation in the reticulocyte cell-free system with native 60S AMV RNA; *b*, immunoprecipitate of an incubation in the reticulocyte lysate with denatured 35S AMV RNA.

*Note added in proof:* We have also succeeded in translating Rauscher leukaemia virus RNA in oocytes. The precursor of the gs antigens pr65 and the processed polypeptides p30 and p15 have been detected.

MARTIN SALDEN
FRED ASSELBERGS
HANS BLOEMENDAL*

*Department of Biochemistry,*
*University of Nijmegen,*
*Geert Grooteplein Noord 21,*
*Nijmegen, The Netherlands*

Received November 24, 1975; accepted January 13, 1976.

* To whom reprint requests should be sent.

1 Gurdon, J. B., Lane, C. D., Woodland, H. R., and Marbaix, G., *Nature*, **233**, 177–182 (1971).
2 Lane, C. D., Marbaix, G., and Gurdon, J. B., *J. molec. Biol.*, **61**, 73–91 (1971).
3 Moar, V. A., Gurdon, J. B., and Lane, C. D., *J. molec. Biol.*, **61**, 93–104 (1971).
4 Berns, A. J. M., Van Kraaikamp, M., Bloemendal, H., and Lane, C. D., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1606–1609 (1972).
5 Kindas-Mügge, I., Lane, C. D., and Keil, G., *J. molec. Biol.*, **87**, 451–462 (1974).
6 Laskey, R. A., Gurdon, J. B., and Crawford, L. V., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3665–3669 (1972).
7 Gurdon, J. B., *Acta Endocr.*, Suppl. **180**, 225–243 (1973).
8 Gielkens, A. L. J., Salden, M. H. L., Bloemendal, H., and Konings, R. N. H., *FEBS Lett.*, **28**, 348–352 (1972).
9 Salden, M. H. L., Gielkens, A. L. J., and Bloemendal, H., *Biochim. biophys. Acta*, **425**, 208–219 (1976).
10 Salden, M. H. L., and Bloemendal, H., *Biochem. biophys. Res. Commun.* **68**, 249–255 (1976).
11 Huebner, R. J., Armstrong, D., Okuyan, M., Sarma, P. S., and Turner, H. C., *Proc. natn. Acad. Sci. U.S.A.*, **51**, 742–750 (1964).
12 Fleissner, E., *J. Virol.*, **8**, 778–785 (1971).
13 Duesberg, P. H., Martin, G. S., and Vogt, P. K., *Virology*, **41**, 631–646 (1970).
14 Vogt, V. M., Eisenman, R., and Diggelmann, H., *J. molec. Biol.*, **96**, 471–493 (1975).
15 Duesberg, P. H., and Robinson, W. S., *Proc. natn. Acad. Sci. U.S.A.*, **55**, 219–227 (1966).
16 Van Zaane, D., Gielkens, A. J. L., Dekker-Michielsen, M. J. A., and Bloemers, H. P. J., *Virology*, **67**, 544–552 (1975).
17 Berns, A., *et. al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 714–718 (1975).
18 Bonner, W. J., and Laskey, R. A., *Eur. J. Biochem.*, **46**, 83–88 (1974).

V - 3

# KINETICS OF SYNTHESIS AND PROCESSING OF PRECURSOR POLYPEPTIDES OF MURINE LEUKEMIA VIRUS IN FROG OOCYTES FOLLOWING MICROINJECTION OF VIRAL RNA

Fred A.M. Asselbergs , Martin H.L. Salden and Hans Bloemendal

ABSTRACT

Maximal rate of translation of 70S virion R-MuLV RNA is observed 10 h after injection into Xenopus oocytes. The viral RNA appears to be functionally stable in the oocytes. After a 1 h labeling period the predominant translation products are three polypeptides, pr $75^{gag}$, pr $77^{gag}$ and pr $65^{gag}$. After longer labeling periods first pr $65^{gag}$, which is the most stable precursor polypeptide, is most prominent. In addition, several intermediates of 35-60 kDalton are observed. Finally, after about 24 h, mature viral core proteins appear. The rate of synthesis of pr $75/77^{gag}$ decreases gradually after injection, suggesting that viral core polypeptides somehow regulate processing or synthesis of the gag-precursors. A heterogeneous group of 90-95 kDalton polypeptides appear to be early processing products of pr $75/77^{gag}$. It will be discussed why no envelope-related polypeptides are synthesized, when viral RNA (70S or 35S) is injected into the cytoplasm or the nucleus of Xenopus oocytes.

Abbreviations:

| | | |
|---|---|---|
| R-MuLV | : | Rauscher murine leukemia virus |
| Mo-MuLV | : | Moloney murine leukemia virus |
| gag | : | group-specific antigen |
| env | : | envelope |

## Introduction

Microinjection of heterologous mRNA into Xenopus laevis oocytes followed by identification of the translation products has been achieved for many species of mRNA both of cellular (reviews: Gurdon, 1974; Lane and Knowland, 1974) and viral origin (Laskey, 1972; Knowland, 1974; Hunter et al., 1976; Rutgers et al., 1976; Salden et al., 1976a and b; DeRobertis et al., 1977; Schwinghamer et al., 1977; Semancik et al., 1977; Van Vloten-Doting et al., 1977; Van Zaane et al., 1977; Nusse et al., 1978; Reynolds et al., 1978). The ability to translate exogenous mRNA renders amphibian oocytes a very suitable model system to study the translation of specific mRNAs under the conditions existing within a living cell. However, quantitative studies on mRNA translation in oocytes have primarily been performed with purified mRNA species of cellular origin (Gurdon et al., 1971; Moar et al., 1971; Giglioni et al., 1973; Lane et al., 1973; Gurdon et al., 1973; Chan et al., 1976; Maniatis et al., 1976; Asselbergs et al., 1978, 1979) and remarkably few quantitative data are available on the translation of viral RNA (Laskey et al., 1977).

The virion RNAs of avian myeloblastosis virus (Salden et al., 1976a; Ghysdael et al., 1977 a and b), Rauscher murine leukemia virus (R-MuLV) (Salden et al., 1976b), Abelson murine leukemia virus (Reynolds et al., 1978) and mouse mammary tumor virus (Nusse et al., 1978), when injected into Xenopus oocytes, direct the synthesis of precursor polypeptides of the virus core proteins or group-specific antigens (gag). The same gag-polypeptides are synthesized after injection of genome-size (35S) mRNA isolated from the polyribosomes of R-MuLV producing JLS-V9 cells (Van Zaane et al., 1977). Only a 22S subgenomic mRNA from JLS-V9 cells is

able to direct the synthesis of R-MuLV envelope-polypeptides in oocytes (Van Zaane et al., 1977). One of the attractive features of the oocyte system is that many of the normal post-synthetic modifications of the primary translation products, which do not occur in cell-free systems, do occur in oocytes (Gurdon, 1974). In this article, we report the kinetics of translation of R-MuLV virion RNA and the kinetics of cleavage of gag-precursor polypeptides into mature viral proteins.

## MATERIALS AND METHODS

### Isolation of RNA

R-MuLV was isolated from the plasma of leukemic mice (Duesberg and Robinson, 1966) and treated with sodium dodecyl-sulphate and pronase. The 70S component of R-MuLV was isolated by centrifugation in isokinetic glycerol gradients (Gielkens et al., 1972). Isolation of rabbit globin and calf crystallin mRNAs has been described previously (Asselbergs et al., 1978).

### Microinjection and culture of Xenopus oocytes

Oocytes were injected with 25 nl of distilled water containing either R-MuLV RNA (12.5 ng), rabbit globin mRNA (5 ng) or calf lens crystallin mRNAs (5 ng) according to procedures previously described (Asselbergs et al., 1978). Two methods of microinjection into the nucleus of the oocytes, described by Gurdon (1976) and Kressmann et al., (1978) respectively, were used as indicated in the text. Oocytes were labeled in modified Barth's medium with 0.5 mCi/ml L-[3,5-$^{3}$H]-leucine (spec. act. 55 Ci/mmol) or L-[2,5-$^{3}$H]-histidine (spec. act. 47 Ci/mmol) or with 0.25 mCi/ml [$^{14}$C] protein hydrolysate (spec. act. 56 mCi/matom C) (all from the Radiochemical Center, Amersham, England). After

labeling for various periods at 19°C, oocytes were washed with cold medium, atypically looking oocytes were removed and oocytes were frozen at -20°C until further processing. In some cases radioactive amino acids were 'chased' by culturing oocytes in medium containing 1 mM of each of 20 amino acids.

Analysis of translation products

Batches of 5-10 oocytes were homogenized in immunoprecipitation buffer (10 mM sodium phosphate buffer pH 7.2 containing 0.9% NaCl, 1.0% Triton-X-100. 0.5% sodium deoxycholate and 0.1% sodium dodecylsulphate. Total and acid precipitable radioactivity of this homogenate was measured (Asselbergs et al., 1978) and indirect immunoprecipitation of R-MuLV proteins was performed in 0.7 ml volumes essentially according to the method of Van Zaane et al., (1975), except that in some cases instead of goat anti-rabbit serum, formaldehyde fixed Staphylococcus aureus bacteria (20 volumes of a 10% suspension per volume of serum) or protein-A-Sepharose (Pharmacia, Uppsala, Sweden) (10 volumes 10% suspension per volume serum) were used as a second precipitating agent (Nusse et al., 1978). The rabbit anti-R-MuLV and anti-$p10^{gag}$ serum (a gift of Dr. J.N. Ihle, Frederick Cancer Centre, Frederick, Md., USA) have been described previously (Reynolds et al., 1970). Anti-$p30^{gag}$ and another batch of anti-total-R-MuLV serum were obtained through courtesy of Dr. J. Gruber, Viral Oncology Program, Natl. Cancer Inst. USA. Immunoprecipitated polypeptides were analyzed on polyacrylamide slab gel gradients (7-18%) (Van Zaane et al., 1975). In some cases indirect immunoprecipitation was used as a quantitative assay using oocyte derived R-MuLV proteins labeled with a different isotope as an internal standard. Direct quantitative immunoprecipitation of calf lens crystallins and the electrophoretic assay of globin synthesis in oocytes have been described previously (Asselbergs et al., 1978).

Culture and labeling of JLS-V9 cells

JLS-V9 cells chronically infected with R-MuLV were cultured and labeled for 15 min with [$^{14}$C] proteinhydrolysate as previously described (Van Zaane et al., 1975).

RESULTS

Time-course of translations of R-MuLV RNA in oocytes

When the 70S RNA complex from R-MuLV, which contains two 35S RNA molecules (Bishop, 1978), is injected into the cytoplasm of Xenopus oocytes, the percentage of R-MuLV polypeptides synthesized increases gradually until about 10 h after injection a constant level of synthesis of the 65 kDalton polypeptide (discussed below) is reached (Fig. 1A). The rate of synthesis of the 75 and 77 kDalton polypeptides decreases after 10 h the percentage of the total incorporation of radioactive amino acids into protein allows a reasonable estimate of the rate of translation of this RNA (Table 1). Of course 1.9 polypeptides mRNA$^{-1}$ h$^{-1}$ is only a *minimum* estimate, since it is based on the assumption that all injected mRNA molecules are translated in the oocyt

The early kinetics of translation of R-MuLV RNA have also been studied by labeling a different batch of oocytes, not for constant periods of time (Fig. 1), but for increasing periods starting immediately after injection of the RNA (Fig. 2). In this experiment a two-phase rise of the percentage R-MuLV protein synthesized is observed (Fig. 2D). A similar biphasic increase is frequently observed with other mRNA species like rabbit globin mRNA or calf $\alpha A_2$-crystallin mRNA (Fig. 3). Compared to the 9S globin mRNA and the 14S $\alpha A_2$-crystallin mRNA, it takes slightly longer before the R-MuLV RNA is 'established' in the translational apparatus of the oocyte.

Table 1. Efficiency of translation of R-MuLV RNA[a]

| | | | |
|---|---|---|---|
| 1 | oocyte protein synthesis[b] | 23 | ng/h, oocyte |
| 2 | total amino acid incorporation[c] | 12,700 | counts/min, h, oocyte |
| 3 | % R-MuLV protein | 2.0 | % |
| 4 | R-MuLV protein synthesis[d] | 0.006S | pmol/h, oocyte |
| 5 | amount of mRNA injected[e] | 0.0036 | pmol/oocyte |
| 6 | efficiency of translation[f] | 1.9 | polypeptides/mRNA, h |

a. Estimates of the efficiency of translation of calf $\alpha A_2$-crystallin and rabbit globin mRNA in oocytes obtained with this method differ less than 15% from the values obtained with the estimation method of Gurdon et al. (1971), which is only applicable to mRNAs of known amino acid composition.

b. Reported estimates of the protein synthesis of Xenopus oocytes range from 19 to 25 ng/h (Davidson, 1976).

c. Oocytes were labeled from 10 to 14 h after injection in 0.25 mCi/ml [$^{14}$C] protein hydrolysate as indicated in the Methods section.

d. Calculated from 1 and 3, assuming an average size of 72 kDalton for the translation product of R-MuLV RNA.

e. 12.5 ng R-MuLV RNA/oocyte.

f. Calculated from 5 and 6. Comparable estimates have been reported for mRNAs encoding rabbit β globin: 7-24 polypeptides/mRNA, h (Gurdon et al., 1971), duck globin: 2 (Lane et al., 1973), chicken ovalbumin: 11 (Chan et al., 1976) and $\alpha A_2$-crystallin: 21 (Asselbergs et al., 1978).

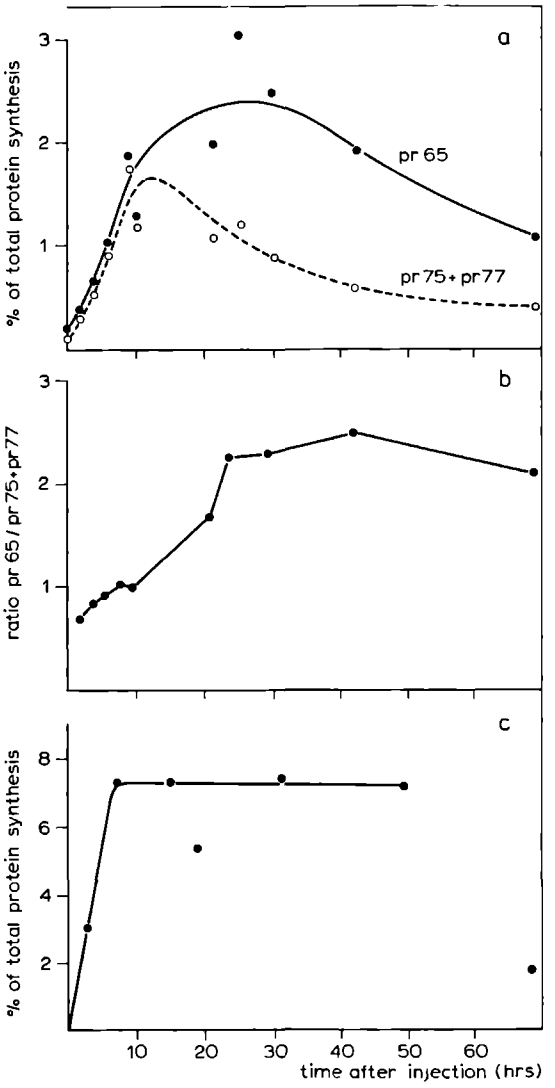

a
% of total protein synthesis
pr 65
pr 75+ pr 77
b
ratio pr 65 / pr 75+pr 77
c
% of total protein synthesis
time after injection (hrs)

Fig. 1 Time-course of translation of R-MuLV RNA in oocytes. (A) Oocytes were microinjected with 12.5 ng R-MuLV RNA each and labeled with [$^{3}$H] leucine for 4 h at various times after injection. The middle of each labeling period is indicated in the figure. The background of aspecific precipitation (about 0.35% of the total incorporation of the oocytes) has not been subtracted, but is indicated by the point at t = 0. Immunoprecipitation with anti total R-MuLV serum was performed as described in the Methods section. A constant amount of homogenate of oocytes, which had been injected with R-MuLV RNA and had been labeled for 24 h with [$^{14}$C] amino acids, was added as an internal standard. Because of the amount of immunoprecipitated [$^{14}$C]-labeled R-MuLV protein was constant, immunoprecipitation of [$^{3}$H]-labeled R-MuLV protein was considered to be proportional to the total amount of [$^{3}$H] labeled R-MuLV protein synthesized by the oocytes. (B) Immunoprecipitates with anti total R-MuLV serum were analyzed on a dodecylsulphate polyacrylamide gel radioactivity of the pr77/75$^{gag}$ and pr65$^{gag}$ regions were measured and the ratio pr77/75:pr65 plotted as a function of the time after RNA injection. (C) Time-course of translation of rabbit globin mRNA in oocytes isolated from the same frog. Globin was labeled with [$^{3}$H] histidine and assayed by polyacrylamide gel electrophoresis (Asselbergs, et al., 1978).

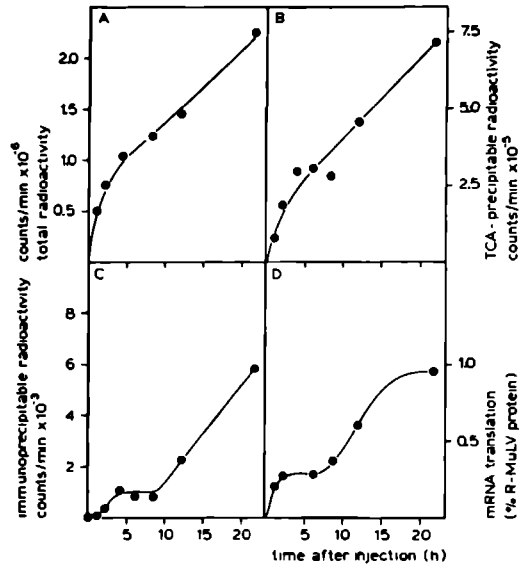



Fig. 2 Early kinetics of synthesis of R-MuLV protein in oocytes injected with 12.5 ng R-MuLV RNA. Oocytes were labeled with [$^{3}$H] leucine immediately after injection for various periods of time. The end of the labeling period is indicated in the figure. Total radioactivity present in oocytes (A) and radioactivity present in total protein (B) is depicted in the figure together with the radioactivity immunoprecipitable with anti total R-MuLV serum. The background of aspecific immunoprecipitation has been subtracted (C). Efficiency of immunoprecipitation was constant as judged from an internal standard (see legend of Fig. 1). In (D) immunoprecipitable radioactivity is expressed as a percentage of total radioactive protein of oocytes. This percentage is considered a measure for translational activity of microinjected R-MuLV RNA.

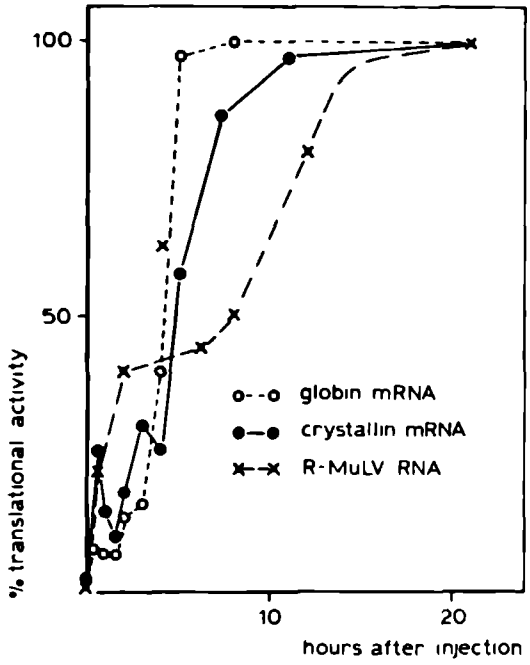



Fig. 3 Comparison of the translational kinetics of different mRNAs. Oocytes were injected with 9S globin mRNA (o- -o), 10-14S calf lens crystallin mRNAs (●—●) or R-MuLV 70S RNA complex (x- -x). The 70S complex contains a 35S translatable RNA species (Bishop, 1978). Oocytes were labeled as described in the legend of Fig. 2 with [$^3$H] leucine in the case of lens mRNA and R-MuLV RNA and with [$^3$H] histidine in the case of globin mRNA. The mRNA activity (% foreign translation product) was measured and expressed as percentage of the mRNA activity found during the longest labeling period. By that time translational activity has reached a constant level (compare Fig. 1 for R-MuLV RNA and for other RNAs; Asselbergs et al., 1979).

Sequential appearance of R-MuLV polypeptides

When oocytes, injected with R-MuLV 70S RNA, are labeled for 0.5 h (not shown) or 1 h (Fig. 4A), 3 translation products with estimated molecular weights of 77, 75 and 65 kDalton are observed. The 77 and 75 kDalton polypeptides were not resolved in our earlier experiments, but instead migrated as a single band of 72 kDalton in the gel (Salden et al., 1976b). A duplicate experiment, in which immunoprecipitation of R-MuLV polypeptides was performed with monospecific antisera against the viral core polypeptides $p30^{gag}$ (Fig. 5B) and $p10^{gag}$ (Fig. 5C), demonstrates that all these polypeptides originate from gag-gene of R-MuLV. During a pulse-chase experiment these high molecular weight precursor polypeptides disappear, while polypeptides of the size of mature viral core proteins appear (Fig. 6). $P10^{gag}$ was not detected in this experiment, since this polypeptide elutes during processing of the gel for fluorography (Van Zaane, 1977). $Pr77^{gag}$, $pr75^{gag}$ and $pr65^{gag}$ are also observed in R-MuLV producing JLS-V9 cells after a 15 min labeling period together with the precursor of the envelope glycoprotein, $pr82^{env}$ (Fig. 4).

When oocytes are labeled for 10 to 22 h starting immediately after injection of the R-MuLV RNA, the most conspicuous translation product is $pr65^{gag}$, while $pr77^{gag}$ and $pr75^{gag}$ gradually disappear as the labeling period increases (Fig. 4, 5). The shift in the ratio pr77 + 75 $^{gag}$ and $pr65^{gag}$ was analyzed in more detail by measuring the radioactivity in the appropriate regions of the gel (Fig. 6A and B). $Pr65^{gag}$ starts to dominate over pr77 + $75^{gag}$ from about 10 h after injection. Less apparent is, that between $pr77^{gag}$ and $pr75^{gag}$, $pr77^{gag}$ tends to dominate during shorter labeling periods (Fig. 4A, 5B), whereas $pr75^{gag}$ dominates after

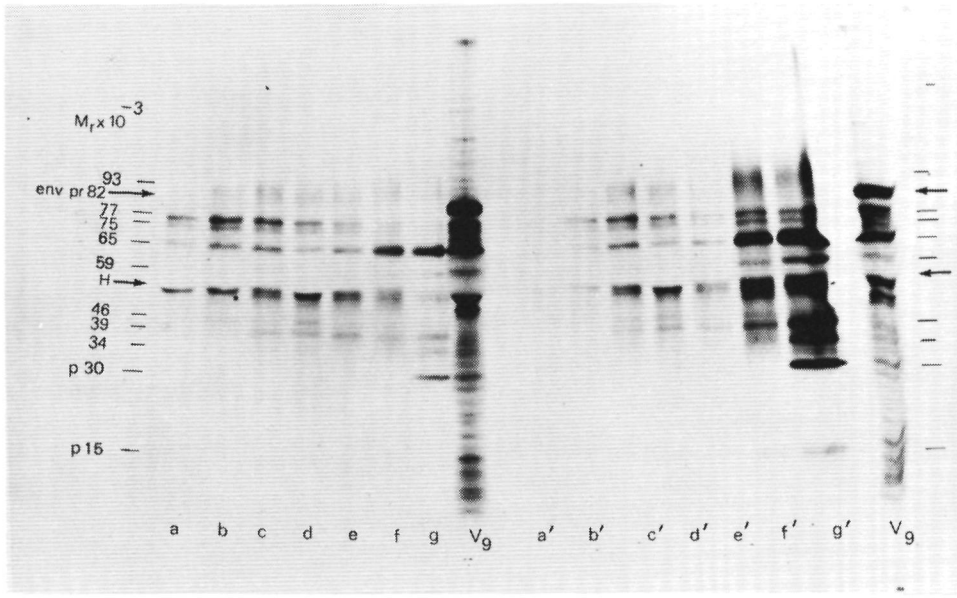



Fig. 4 Sequential appearance of R-MuLV proteins in oocytes microinjected with R-MuLV RNA. Oocytes were labeled as indicated in the legend of Fig. 2 except that no [$^{14}$C] labeled oocytes were included as an internal standard. A constant amount of [$^{3}$H]-labeled protein precipitated with anti total R-MuLV serum (4000 counts/min) was applied onto the gel (a-g) or an amount equivalent to the products synthesized by two oocytes (a'-g'). For comparison an immunoprecipitate of the R-MuLV producing cell line JLS-$V_9$ is included ($V_9$). Positions of envelope precursor polypeptide pr $82^{env}$, the immunoglobulin heavy chain (H) and the viral core proteins $p30^{gag}$ and $p15^{gag}$ are shown together with molecular weights of gag-precursor polypeptides and intermediate processing products. Oocytes were labeled for 1 h (a), 2 h (b), 4 h (c), 6 h (d), 8 h (e), 12 h (f) or 22 h (g).

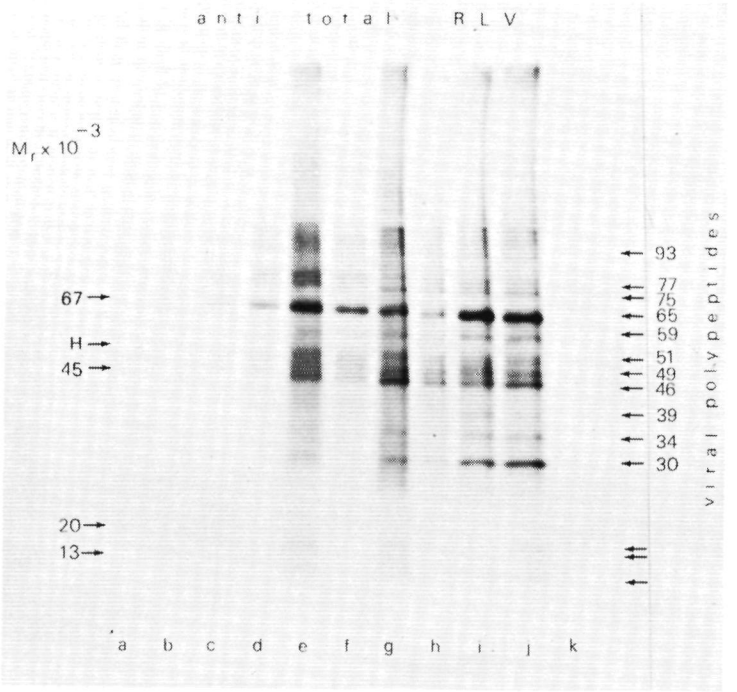
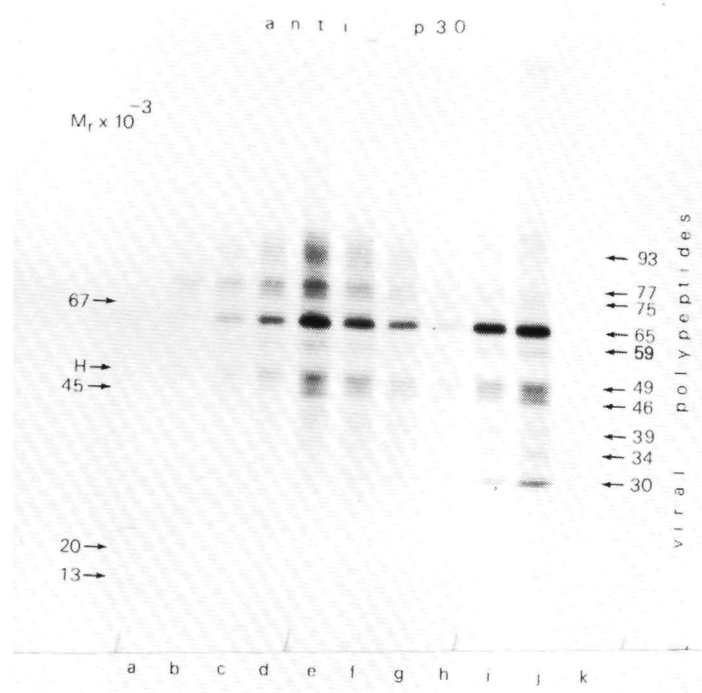
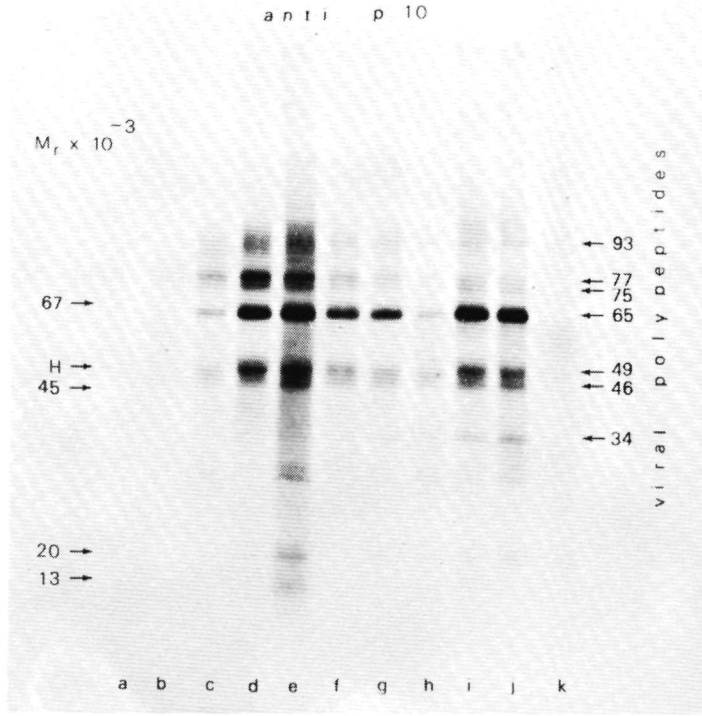



Fig. 5. Immunoprecipitation of R-MuLV precursor polypeptides with monospecific antisera. Oocytes were labeled with [$^{35}$S] methionine and immunoprecipitated with anti total R-MuLV and monospecific antisera against the viral proteins p30$^{gag}$ and p10$^{gag}$. Control oocytes were injected with distilled water and labeled for 4 h (a) or for 20 h (k). Oocytes injected with R-MuLV RNA were labeled for 2 h (b), 4 h(c), 6 h (d), 8 h (e), 10 h (f), 14 h (g), 20 h (h), 30 h (i) and 48 h (j). On the left side of the figure the position of reference proteins bovine serum albumin (67,000 dalton), immunoglobulin heavy chain (H, about 50,000), ovalbumin (45,000), calf $\alpha A_2$-crystallin (20,000) and ribonuclease (13,000) is indicated. At the right side the apparent molecular weights of R-MuLV polypeptides is given. The viral polypeptides p10 and p15 are not labeled, since they do not contain methionine residues.


anti p30
$M_r \times 10^{-3}$
67
H
45
20
13
93
77
75
65
59
49
46
39
34
30
viral polypeptides
a b c d e f g h i j k


anti p10
$M_r \times 10^{-3}$
67
H
45
20
13
93
77
75
65
49
46
34
viral polypeptides
a b c d e f g h i j k

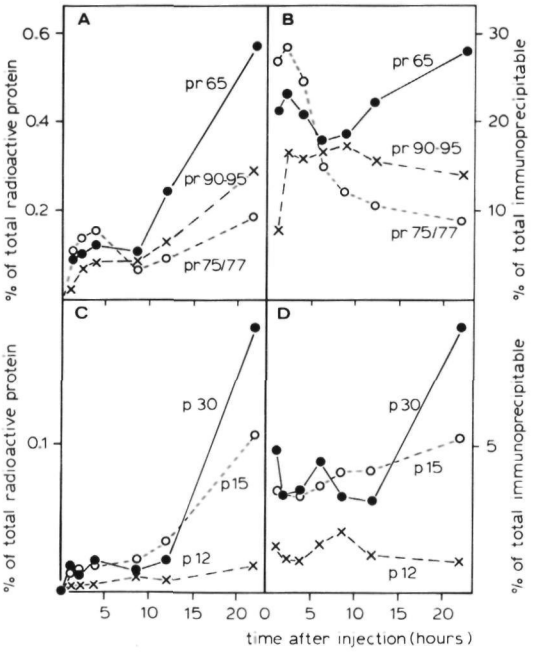



Fig. 6 Labeling kinetics of R-MuLV proteins. Oocytes were labeled as described in the legend to Fig. 2. The immunoprecipitates obtained with anti total R-MuLV serum were analyzed on a polyacrylamide gel as in Fig. 5. Radioactivity of different parts of the gel was measured and values are expressed as a percentage of the total protein radioactivity of oocytes (A, C) or as a percentage of the total R-MuLV immunoprecipitable radioactivity (B, D). In A and B labeling kinetics of three high molecular weight polypeptides are shown. $Pr75/77^{gag}$ (o-●-) and $pr65^{gag}$ (●——●) have previously been identified in oocytes ($pr75/77^{gag}$ in this gel system is identical to the 72,000 dalton gag-precursor previously detected in oocytes; Salden et al., 1976b). The other curve (x- -x) depicts incorporation into a polypeptides of about 93,000 dalton, all products of the gag-gene of R-MuLV (see text). In C and D labeling kinetics of three viral core polypeptides are depicted, $p30^{gag}$ (●——●), $p15^{gag}$ (o- o-) and $p12^{gag}$ (x- -x).

longer periods (Fig. 4G, 5I). In another experiment oocytes were labeled for 4 h periods at various times after injection of the virion RNA Fig. 1). The ratio $pr65^{gag}/pr77+75^{gag}$ increases steadily until it levels off after about 24 h (Fig. 1B).

In addition to the three gag precursor polypeptides mentioned above, another group of high molecular weight translation products is observed. These 90-95 kDalton polypeptides, which under certain conditions may constitute as much as 15% of the labeled R-MuLV polypeptides (Fig. 7B), migrate as a smear of poorly resolved bands in the gel (Fig. 4, 5). This phenomenon is known for glycoproteins (Van Zaane et al., 1975). However, the incorporation of radioactive glucosamine or fucose in oocytes was too small to establish, whether they really are glycoproteins. The 90-95 kDalton polypeptides can be precipitated with antisera against the viral core proteins $p30^{gag}$ and $p10^{gag}$ (Fig. 5B, C), but not with antiserum against the envelope glycoprotein $gp70^{env}$ (not shown), and they will for convenience be designated $pr90\text{-}95^{gag}$. During a pulse-chase experiment $pr90\text{-}95^{gag}$ disappear in a similar manner as $pr75^{gag}$ and $pr65^{gag}$ (Fig. 6, 8). From short labeling experiments it appears that the 90-95 kDalton polypeptides appear somewhat later than the other gag-precursors (Fig. 7A, B).

When oocytes are labeled for increasing periods, mature $p30^{gag}$ and $p15^{gag}$ appear after about 24 h (Fig. 4, 5, 7). $P12^{gag}$ appears somewhat later, since it is not detected after pulse-labeling for 24 h, but it appears during the chase period (Fig. 6). Quantitation of the fluctuating amounts of R-MuLV polypeptides demonstrates the existence of a temporal gap between the disappearance of the gag-precursors (here defined as polypeptides of 65 kDalton and larger) and the appearance of

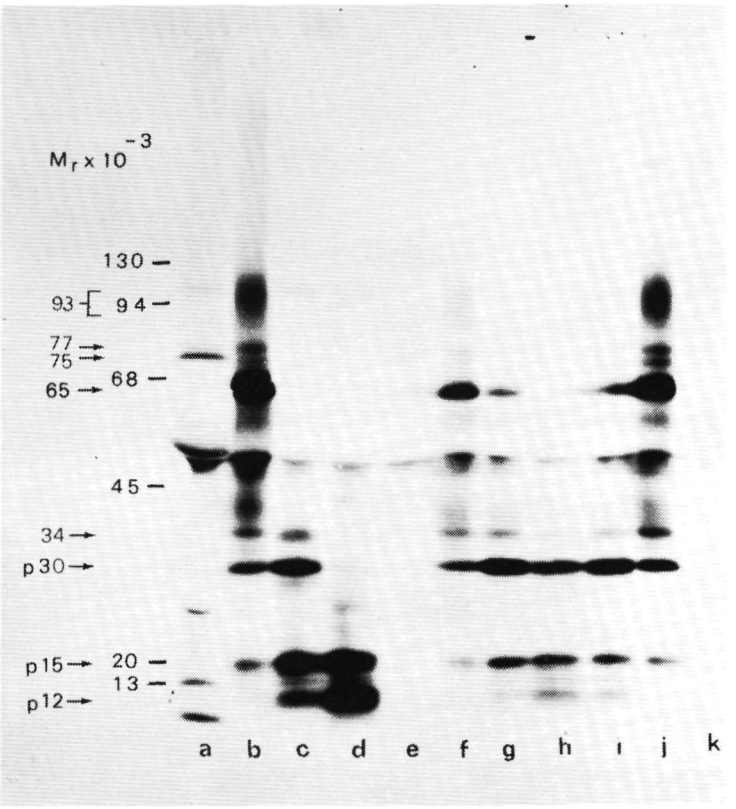



Fig. 7 Pulse-chase analysis of R-MuLV polypeptides synthesized in oocytes. Oocytes from two different frogs (a-d and e-k) were labeled as indicated in the legend to Fig. 7. Oocytes were injected with 25 nl distilled water (a, e, k) or with 12.5 ng R-MuLV RNA (b-d, f-j). Oocytes were labeled for 24 h (a, b, e, f) and chased with unlabeled amino acids for 1 day (g), 2 days (c, h), 4 days (j, k) and 5 days (d).

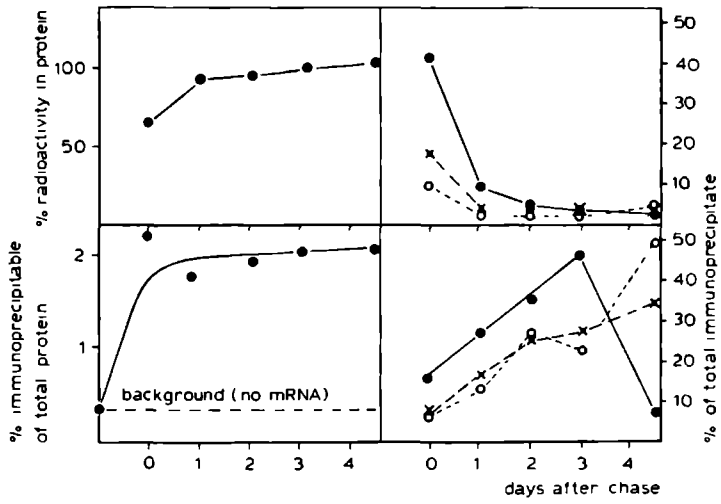



Fig. 8 Pulse-chase kinetics of R-MuLV polypeptides in oocytes. Oocytes were injected with R-MuLV RNA, labeled for 24 h with [$^{14}$C] amino acids and then chased in fresh medium containing non-radioactive amino acids. Total oocyte radioactivity and radioactivity present in protein were measured. The % radioactive protein is depicted in (A). Immunoprecipitable radioactivity using anti total R-MuLV serum is expressed as a percentage of total protein radioactivity (B). Immunoprecipitates were also analyzed on a polyacrylamide gel slab (shown in Fig. 7) and the radioactivity of the appropriate regions of the gel was measured and expressed as % of total immunoprecipitable radioactivity. Curves of three high molecular weight R-MuLV polypeptides, pr75/77$^{gag}$ (o- -o), pr65$^{gag}$ (●—●) and the polypeptide(s) of about 93,000 dalton (x- -x), are depicted in (C). Kinetics of synthesis of three mature viral proteins, p30$^{gag}$ (●—●), p15$^{gag}$ (o- -o) and p12$^{gag}$ (x- x), are shown in (D). The data shown in this figure were compiled from experiments with oocytes from two frogs.

mature viral proteins (Fig. 8), indicating the existence of a number of rather stable processing intermediates of 35-60 kDalton (Fig. 4, 5).

## DISCUSSION

### Synthesis and processing of the gag-precursor polypeptides

Three genetic domains have been identified in the genome of MuLV encoding the viral core polypeptides (also called group-specific-antigens), the RNA dependent DNA polymerase and the envelope protein respectively. The gag, pol-and env-genes occur in this 5' to 3' sequence in the viral RNA genome (review, Bishop, 1978). In a cell-free system the 35S RNA is translated mainly into precursors of the core polypeptides, while as minor translation products precursors containing both the core polypeptides and the reverse transcriptase occur (review, Eisenman and Vogt, 1978). The latter are thought to arise as a result of a low frequency of readthrough of the stop-codon at the end of the gag-gene (Salden et al., 1976b). The translation products of virion MuLV RNA, listed in Table 2, are with one exception all related to the gag-gene. The only polypeptide not precipitable with antisera against $p30^{gag}$ and $p10^{gag}$ is a minor 51 kDalton translation product, which only precipitates with antiserum raised against whole MuLV (Fig. 5A, Table 2). It has not been possible to demonstrate beyond doubt the synthesis in oocytes of the MuLV 180 kDalton gag-pol-polypeptide due to the frequent occurrence in our immunoprecipitates of an aspecifically precipitating oocyte polypeptide of similar size (for example Fig. 9). However, a metabolically instable minor translation product of 170 kDalton (slightly smaller than the coprecipitating oocyte protein) presumably identical to the gag-pol-polypeptide, has been observed after injection of viral RNA of other

retroviruses, namely avian myeloblastosis virus (Asselbergs and Salden, unpublished results) and bovine leukemia virus (Ghysdael et al., 1979). It is therefore, not unlikely that also the MuLV gag-pol polypeptide is synthesized in oocytes.

$Pr65^{gag}$ (Table 2d) contains the complete amino acid sequences of all four R-MuLV core polypeptides (Barbacid, et al., 1976), while the larger precursor $pr76^{gag}$ (probably identical to $pr77^{gag}$ or $pr75^{gag}$ in oocytes) contains an additional peptide, here designated X (Table 2b, c), at the $NH_2$-terminal end (Schultz and Oroszlan, 1978). Both $pr65^{gag}$ and $pr76^{gag}$ can be labeled in a cell-free system with $tRNA_f^{Met}$ (Edwards and Fan, 1979), but only termination of $pr76^{gag}$ can be suppressed by addition of yeast amber mutant tRNA to the translation system (Phillipson et al., 1978). It thus appears that $pr76^{gag}$ and $pr65^{gag}$ are translated from different 35S mRNAs. Probably due to its $NH_2$-terminal extension $pr76^{gag}$ appears to follow a different pathway in the cell. $Pr76^{gag}$ isolated from infected cells appears to be glycosylated and to be processed into polypeptides with higher degree of glycosylation (Edwards and Fan, 1979), similar to the 90-95 kDalton gag polypeptides observed in oocytes (fig. 4). In our experiments $pr76^{gag}$ migrates as a double band in the gel. The slower migrating polypeptide, $pr77^{gag}$, appears to be the precursor of $pr75^{gag}$, since it tends to dominate during short labeling periods (Fig. 4,5). In a reticulocyte cell-free system membrane bound polyribosomes isolated from JLS-V9 cells direct the synthesis of a slightly larger polypeptide than free polyribosomes (Gielkens, et al., 1976). It is therefore also possible that $pr77^{gag}$ differs from $pr75^{gag}$ in a post-synthetic modification, most likely a glycosylation. A gag-precursor polypeptide of 82 kDalton, is synthesized in the presence of

Table 2. R-MuLV polypeptides synthesized in oocytes.

| | JLS-$V_9$ 15 min. pulse labeling. | tentative identification | apparent molecular weight (kDalton) | |
|---|---|---|---|---|
| a. | | ?-x-p15-p12-p30-p10-? | 90-95 | Polypeptides appearing |
| b. | + | ?-x-p15-p12-p30-p10 | 77 | within 2 h after injection |
| c. | + | x-p15-p12-p30-p10 | 75 | of RNA and disappearing in about |
| d. | + | p15-p12-p30-p10 | 65 | 24 h during pulse-chase exp. |
| e. | + | p15-p12-p30 | 59 | |
| f. | | | 51 | |
| g. | | ?-p12-p30-p10 | 49 | |
| h. | | p12-p30-p10 | 46 | |
| i. | | p12-p30 | 39 | |
| j. | | p30-p10 | 34 | |
| k. | | p30 | 30 | Viral polypeptides appear |
| l. | | p15 | 18 | about 20 h after injection |
| m. | | p12 | 12 | of RNA; radioactivity still |
| n. | | p10 | 8 | increases 3 days after chase. |

Subscripts to Table 2:

This table compiles the results from figures 4 and 5. The tentative identification is based on the known sequence of the MuLV core polypeptides within the gag precursor pr65 (Barbacid, et al., 1976). The presence of the polypeptide sequences of the underlined core polypeptides $p30^{gag}$, $p12^{gag}$ and $p10^{gag}$ has been demonstrated by immunoprecipitation with monospecific antisera against $p30^{gag}$ and $p10^{gag}$ (Fig. 5) and $p12^{gag}$ (Reynolds, et al., 1978). The polypeptide $p10^{gag}$ was not detected in the experiment shown in Figures 4 and 5, since p10 elutes from the gel during processing for fluorography (Van Zaane, 1977). The nomenclature of MuLV polypeptides is based on their apparent molecular weights during gel filtration in the presence of guanidine-HCL, which is different from the apparent molecular weight in sodium dodecylsulphate polyacrylamide gels. Identical apparent molecular weights were observed for $p30^{gag}$, $p15^{gag}$, $p12^{gag}$ and $p10^{gag}$ regardless whether derived from MuLV virions, JLS-V9 cells or oocytes (not shown).

an arginine analog canavanine in JLS-V9 cells (Van Zaane, et al., 1976) and in a cell-free system (Salden, et al., 1976b). Therefore, the possibility that both $pr76^{gag}$ and $pr65^{gag}$ are processing products of 82 kDalton primary translation products should be kept in mind. The largest (group of) translation product(s) of R-MuLV detected in oocytes, $pr90\text{-}95^{gag}$ (Table 2a), seems to appear slightly later than $pr77^{gag}$, $pr75^{gag}$ and $pr65^{gag}$ (Fig. 7), suggesting that $pr90\text{-}95^{gag}$ is not a primary translation product of R-MuLV RNA. An unstable gag processing intermediate of about 90 kDalton (JLS-V9 $pr90^{gag}$ has also been observed in JLS-V9 cells (Van Zaane, et al., 1976). If $pr82^{gag}$ is the primary translation product of the gag-region, the JLS-V9-$pr90^{gag}$ and the oocyte $pr90\text{-}95^{gag}$ might be derived from $pr82^{gag}$, possibly via $pr76^{gag}$, by incorporation of sugar residues. Such glycosylated forms of the gag precursor have been detected on the cell surface of MuLV infected cells under certain conditions (Evans, et al., 1977; Ledbetter, et al., 1977; Edwards and Fan, 1979). However the possibility that JLS-V9 $pr90^{gag}$ and oocyte $pr90\text{-}95^{gag}$ are processing products of the gag-pol-precursor cannot be excluded.

The tentative identification of the processing intermediates (Table 2e, g-i) is based on the assumption that all have been derived from $pr65^{gag}$. The sequence of the viral core polypeptides within this precursor has been established by Barbacid et al., (1976). Indirect evidence had prompted them to conclude that the positions of $p10^{gag}$ and $p30^{gag}$ within the precursor must be adjacent. Their conclusion is confirmed by the discovery of the small polypeptide $pr34^{gag}$ in oocytes (Table 2j), which contains the antigenic determinants of both $p10^{gag}$ and $p30^{gag}$. The difference between $pr49^{gag}$, which appears only early after

injection of R-MuLV RNA (Fig. 4A, D) and pr46$^{gag}$, which appears slightly later (Fig. 4C,G) is not clear.

Viral polypeptides have been implicated in the processing of polyproteins of several viruses (Esteban and Kerr, 1974; Scupham, et al., 1977; Pelham, 1978). The core protein pr15$^{gag}$ of Rous sarcoma virus, equivalent to MuLV p12$^{gag}$, has been identified as a proteolytic enzyme involved in the processing of the gag-precursor, that is: its own precursor (von der Helm, 1977). A proteolytic factor capable of cleaving the gag-precursor has also been isolated from MuLV virions (Yoshinaka and Luftig, 1977a and b). However the processing of the gag-precursor in oocytes proceeds initially in complete absence of viral proteins. There is apparently a shift in the processing pathway of pr65$^{gag}$ which possibly may be caused by viral translation products. Processing intermediate pr49$^{gag}$ is only detectable up to 6 h after injection, whereas later pr46$^{gag}$ appears (Fig. 4).

Kinetics of translation of MuLV RNA in oocytes

We have previously demonstrated that the gradual increase of the (apparent) rate of translation of microinjected mRNAs is accompanied by a parallel decrease of the sensitivity to competitive inhibition of translation by a second mRNA injected afterwards up to the level of the oocyte endogenous mRNAs. The injected mRNA is by this criterion gradually established as an 'endogenous' mRNA (Asselbergs et al., 1979). The slower rate of establishment of 29S vitellogenin mRNA in respect to 9S globin and 16S albumin mRNA has been attributed to the larger size of vitellogenin mRNA (Berridge and Lane, 1976). However, the rate of

establishment of R-MuLV RNA is only slightly slower, than that of lens and globin mRNA (Fig. 3), despite the enormous difference in the size of the RNA molecules, the 35S MuLV RNA genome being about 3.4 x $10^6$ Dalton (Riggin et al., 1975), 14S $\alpha A_2$-crystallin mRNA 520,000 Dalton and 9S globin mRNA 200,000 Dalton (Berns et al., 1974).

Microinjected rabbit globin mRNA is completely stable in oocytes for periods up to two weeks (Gurdon et al., 1973), whereas typical unstable mRNAs, such as poly(A)-free globin mRNA or histone mRNAs, are no longer translated 1 day after injection (Huez et al., 1978). In our experiment the $pr65^{gag}$ mRNA appears to be a reasonably stable mRNA, since it is still translated 3 days after injection (Fig. 1A). The translation of the mRNA(s) encoding $pr77^{gag}$ and $pr75^{gag}$ appears to increase parallel to the translation of the $pr65^{gag}$ mRNA up to 10 h after injection (Fig. 1A). Between 10 and 25 h after injection the relative rate of synthesis of $pr65^{gag}$ compared to the rate of synthesis of pr77 and $75^{gag}$ increases dramatically, whereas between 25 and 60 h after injection the relative rates of synthesis are constant again (Fig. 1B). These differential rates of synthesis of the gag precursor polypeptides may reflect a regulatory effect by the mature viral polypeptides, which start to accumulate between 12 and 24 h after injection (Fig. 6) on the mRNA translation or on the processing of the precursor polypeptides.

Microinjection of MuLV RNA into the oocyte nucleus

One of the intriging properties of the viral RNA of the murine leukemia viruses is that the 35S RNA molecule, although it contains the genetic information for the synthesis of the envelope proteins, does not express this information, when the viral RNA (or the 35S mRNA isolated

from JLS-V9 polyribosomes) is translated in a cell-free system or in oocytes (Salden et al., 1976a; Gielkens et al., 1976). In a cell-free system a 22S mRNA isolated from the polyribosomes of R-MuLV producing JLS-V9 cells directs the synthesis of a (probably unglycosylated) envelope polypeptide of 70 kDalton (Gielkens et al., 1976). When injected in the cytoplasm of Xenopus oocytes, this subgenomic mRNA directs the synthesis of envelope precursor pr $82^{env}$ and of mature $gp70^{env}$ (Van Zaane, et al., 1977). The nucleotide sequences at the 5' end and at the 3' end of the 22S mRNA are identical to those found in the 35S RNA (Rothenberg et al., 1977). Therefore the 22S mRNA is probably derived from a genome size primary transcript of the integrated viral genome by removal of some intervening sequences, a process now generally referred to as 'splicing'. Since most of the processing of mRNA occurs in the nucleus, we decided to inject 35S RNA directly into the oocyte nucleus.

Technically, intranuclear microinjection is complicated by the opaqueness of the cytoplasm of the oocyte due to the yolk platelets. One way of hitting the nucleus is to aim the microinjection needle precisely at the animal pole of the oocyte directly under which the germinal vesicle (the giant oocyte nucleus) lies hidden (Gurdon, 1976). Alternatively one can visualize the nucleus by centrifuging the oocyte at low speed. The nucleus, which has a lower density than the cytoplasm, floats towards the surface of the cell, where it displaces the pigment granules creating a lighter spot surrounded by a dark ring at the surface of the oocyte. Microinjection is aimed at the centre of the ring (Kressmann et al., 1977). Transcription of DNA inserted into the nucleus by these techniques and subsequent coupled translation of the synthesized mRNA (implying also correct transport and splicing of mRNA precursors)

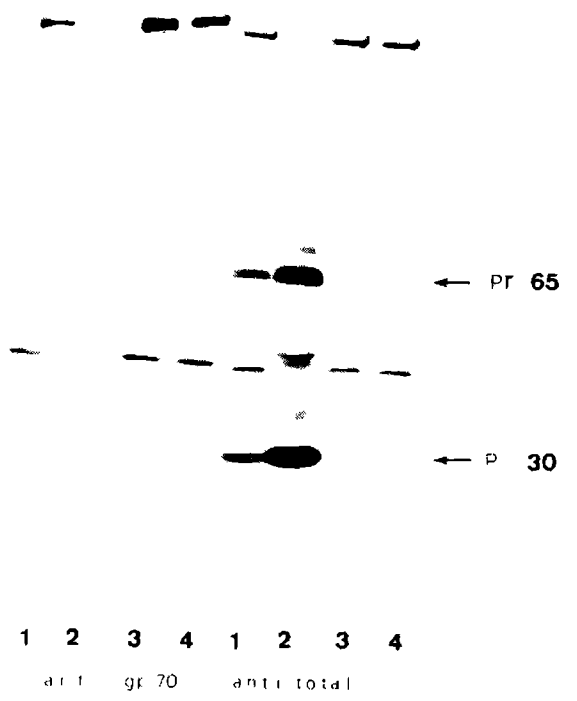



Fig. 9 Microinjection of Mo-MuLV RNA into the nucleus of Xenopus oocytes. 15 nl Mo-MuLV 35S virion RNA (1 mg/ml) (1, 2) or distilled water (3, 4) was injected into the nucleus (1, 3) or the cytoplasm (2, 4) of 20 Xenopus oocytes which were subsequently labeled for 24 h. Immunoprecipitation was carried out with antiserum against Mo-MuLV gp70 (left) or antiserum against whole Mo-MuLV virions (right). A portion of the immunoprecipitates equivalent to 4 oocytes was analyzed on the slab gel.

has been demonstrated for SV40 DNA and cloned Drosophila histone DNA (DeRobertis et al., 1977; Rungger and Türler, 1978) and (restriction fragments) of adenovirus DNA (F.A.M.A. and M.H.L.S., unpublished results obtained in collaboration with A. van der Eb). Using either technique we were unable to detect the synthesis of envelope polypeptides after intranuclear injection of any form of virion RNA. Both with 35S RNA of Moloney MuLV (Fig. 9) or Rauscher MuLV (not shown) and with 70S R-MuLV exclusively synthesis of gag-polypeptides is observed. The amount of gag-related polypeptides synthesized after injection of viral RNA into the nucleus is lower as compared with injection into the cytoplasm. Injection of whole virus (R-MuLV or Mo-MuLV) solubilized by freezing and thawing or with 0.4% Nonidet P-40 results in the exclusive synthesis of the gag-polypeptides.

Our observations contrast with the results obtained by microinjection of Rous sarcoma viral RNA into the nucleus of tissue culture cells (Stacey and Hanafusa, 1978). However in the latter experiments the possibility of recombination at the DNA-level cannot totally be excluded, since the injected tissue culture cells were preinfected with a mutant virus capable of synthesizing reverse transcriptase (but not envelope protein). As to why oocytes apparently fail to splice 35S RNA, no definite explanation is available at present. It might be due to deficiencies in the 35S virion RNA (for example because it is internally methylated (Bondurant, et al., 1976)) or in the oocytes. Differences between amphibian and mammalian cells (for example the incubation temperature of oocytes is 19$^{o}$C) apparently do not affect splicing of mammalian mRNA in oocytes very much, since synthesis of the spliced mRNA

encoding SV40 T-antigen has been detected after injection of SV40 DNA into oocytes (Rungger and TÜrler, 1978). Perhaps splicing is in some intricate way connected to the transcription process or it may involve a virus encoded protein that is not synthesized in oocytes. Other possibilities include a deficiency in the transport of the env-mRNA to the cytoplasm or insufficient stabilization of the env-mRNA in oocytes.

ACKNOWLEDGEMENTS

The present investigations have partly been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid of the Netherlands Organization for the Advancement of Pure Research (ZWO). We are grateful to Mr. Chris Saris for a gift of 35S MoMuLV RNA and to Ms Annemiete van der Kemp for analysis of fig. 9.

REFERENCES

Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal H. (1978). Synthesis of lens crystallins in Xenopus oocytes as determined by quantitative immunoprecipitation. Eur. J. Biochem. 87, 517-524.

Asselbergs, F.A.M., van Venrooij, W.J. and Bloemendal, H. (1979). Messenger RNA competition in living Xenopus oocytes. Eur. J. Biochem., in press.

Barbacid, M., Stephenson, J.R. and Aaronson, S.A. (1976). Gag-gene of mammalian type C RNA tumor viruses. Nature 262, 554-559.

Berns, A., Janssen, P. and Bloemendal, H. (1974). The molecular weight of the 14S calf lens messenger RNA. Biochem. Biophys. Res. Commun. 59, 1157-1164.

Berridge, M.V. and Lane, C.D. (1976). Translation of Xenopus liver mRNA in Xenopus oocytes; vitellogenin synthesis and conversion to yolk platelet proteins. Cell 8, 283-297.

Bishop, J.M. (1978). Retroviruses. Ann. Review of Biochemistry. 47, 85-88.

Bondurant, M. Hashimoto, S.I. and Green, M., (1976). Methylation pattern of genome RNA from Moloney Leukemia virus. J. Virol. 19, 998-1005.

Chan, L., Kohler, P.O. and O'Malley, B. (1976). Translation of ovalbumin mRNA in Xenopus laevis oocytes: characterization of the system and effects of estrogen on injected mRNA populations. J. Clin. Invest. 57, 576-585.

Davidson, E.M. (1976). Gene activity in development, 2nd edition. Acad. Press., New York.

DeRobertis, E.M. and Mertz, J.E. (1977). Coupled transcription-translation of DNA injected into Xenopus oocytes. Cell 12, 175-182.

DeRobertis, E.M., Partington, G.A., Longthorne, R.F. and Gurdon, J.B. (1977). Somatic nuclei in amphibian oocytes; evidence for selective gene expression. Embryol. Exp. Morphol. 40, 199-214.

Duesberg, P.H. and Robinson, W.S. (1966). Nucleic acid and protein isolated from the Rauscher mouse leukemia virus (MLV). Proc. Natl. Acad. Sci. USA 55, 219-227.

Edwards, S.A. and Fan, H. (1979) gag-Related polyproteins of Moloney Murine Leukemia Virus: Evidence for independent synthesis of glycosylated and unglycosylated forms. J. Virol. 30, 551-563.

Eisenman, R.N. and Vogt, V.M., (1978). Biosynthesis of oncovirus proteins. Biochim. Biophys. Acta. 473, 188-239.

Esteban, M. and Kerr, I.M. (1974). The synthesis of encephalomyocarditis virus polypeptides in infected L-cells and cell-free systems. Eur. J. Biochem. 45, 567-576.

Evans, L.H., Dressler, S. and Kabat, D. (1977). Synthesis and glycosylation of polyprotein to the internal core protein of Fried murine leukemia virus. J. Virol. 24, 865-874.

Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D.P., Burny, A. Cleuter, Y. Huez, G., Kettman, R., Marbaix, G., Portetelle, D. and Chantrenne, H. (1977a). Frog oocytes synthesize and completely process the precursor polypeptide to virion structural proteins after mircoinjection of avian myeloblastosis virus. Prc. Natl. Acad. Sci. USA 74, 3230-3234.

Ghysdael, J., Hubert, E., Travnicek, M., Bolognesi, D.P., Cleuter, Y., Huez, G., Marbaix, G., Portetelle, D. and Chantrenne, H. (1977b). Synthesis and complete processing of a high molecular weight precursor polypeptide to viral structural proteins in toad oocytes microinjected with avian myeloblastosis virus ribonucleic acid. Biochem. Soc. Trans. 5, 950-953.

Gielkens, A.J.L., Salden, M.H.L., Bloemendal, H. and Konings, R.N. H. (1972). Translation of onocogenic viral RNA and eukaryotic messenger RNA in the E coli cell-free system. FEBS. Lett. 20, 348-352.

Gielkens, A.J.L., van Zaane, D., Bloemers, H.P.J. and Bloemendal, H. (1976). Synthesis of Rauscher murine leukemia virus specific polypeptides in vitro. Proc. Natl. Acad. Sci. USA 73, 356-360.

Giglioni, B., Gianni, A.M., Ottolenghi, S. and Rungger, D. (1973). Translational control of globin synthesis by haemin in Xenopus oocytes. Nature New Biol. 246, 99-102.

Gurdon, J.B. (1974). Molecular biology in a living cell. Nature 248, 772-776.

Gurdon, J.B. (1976). Injected nuclei in frog oocytes: fate, enlargement and chromatin dispersal. J. Embryol. Exp. Morphol. 36, 523-540.

Gurdon, J.B., Lane, C.D., Woodland, H.R. and Marbaix, G., (1971). Use of frog egges and oocytes for the study of messenger RNA and its translation in living cells. Nature 233, 177-182.

Gurdon, J.B. Lingrel, J.B. and Marbaix, G. (1973). Message stability in injected oocytes: long life of mammalian $\alpha$ and $\beta$ globin messages. J. Mol. Biol. 80, 539-551.

Huez, G., Marbaix, G., Gallwitz, D., Weinberg, E., Devos, R., Hubert, E. and Cleuter, Y. (1978) Functional stabilization of HeLa cell histone messenger RNAs injected into Xenopus oocytes by 3-OH polyadenylation Nature 271, 572-573.

Hunter, T.R., Hunt, T., Knowland, J., and Zimmern, D. (1976). Messenger RNA for the coat protein of tobacco mosaic virus. Nature 260, 759-764.

Knowland, J. (1974). Protein synthesis directed by the RNA from a plant virus in a normal animal cell. Genetics 78, 383-394.

Kressmann, A., Clarkson, S.G., Telford, J.L. and Birnstiel, M.L. (1977). Transcription of Xenopus tDNA met and sea urchin histone DNA injected into the Xenopus oocyte nucleus. Cold Spring Harbor Symp. Quant. Biol. 42, 1077-1082.

Lane, C.D., Gregory, C.M. and Morel, C. (1973). Duck haemoglobin synthesis in frog cells: the translation and assay of reticulocyte 9-S RNA in oocytes of Xenopus laevis. Eur. J. Biochem. 34, 219-227.

Lane, C.D. and Woodland, J.S. (1974). The injection of RNA into living cells; the use of frog oocytes for the assay of mRNA and the study of the control of gene expression. In: Biochemistry of development, Vol. 3 (R. Weber, ed.). pp. 145-181, Academic Press, New York.

Laskey, R.A., Gurdon, J.B. and Crawford, L.V. (1972). Translation of Encephalomyocarditis viral RNA in oocytes of Xenopus laevis. Proc. Natl. Acad. Sci USA. 69, 3665-3669.

Laskey, R.A., Mills, A.D., Gurdon, J.B. and Partington, G.A. (1977). Protein synthesis in oocytes of Xenopus laevis is not regulated by the supply of mRNA. Cell 11, 345-352.

Ledbetter, J., Nowinski, R.C. and Emery, S. (1977). Viral proteins expressed on the surface of murine leukemia cells. J. Virol. 22, 65-73.

Maniatis, G.M., Ramirez, F., Cann, A., Marks, P. and Bank, A. (1976). Translation and stability of human globin mRNA in Xenopus oocytes. J. Clin. Invest. 58 1419-1427.

Marbaix, G. and Gurdon, J.B. (1972). The effect of reticulocyte "ribosome" factors on the translation of hemoglobin messenger RNA in living frog oocytes. Biochim. Biophys. Acta. 281, 86-92.

Moar, V.A., Gurdon, J.B. Lane, C.D. and Marbaix, G. (1971). Translational capacity of living frog eggs and oocytes as judged by messenger RNA injected. J. Mol. Biol. 61, 93-104.

Nusse, R., Asselbergs, F.A.M., Salden, M.H.L., Michalides, R.J.A.M. and Bloemendal, H., (1978). Translation of mouse mammary tumor virus RNA precursor polypeptides are phosphorylated during processing. Virology 21, 106-115.

Oroszlan, S., Schultz, A.M., Henderson, L.E. and Olpin, J.L. (1979). Biochemical and primary structure studies of retrovirus of retrovirus gag-polyproteins. Proc. Natl. Acad Sci. USA. 76, in press.

Pelham, H.R.B. (1978). Translation of encephalomyocarditis virus in vitro yields an active proteolytic processing enzyme. Eur. J. Biochem. 85, 457-462.

Reynolds, R.K., Van de Ven, W.J.M., and Stephenson, J.R. (1978). Translation of type C viral RNAs in Xenopus oocytes; Evidence that the 120,000 molecular weight polyprotein expressed in Abelson leukemia virus transformed cells is viral coded. J. Virol. 25, 665-670.

Riggin, C.H., Bondurant, M. and Mitchell, W.M. (1975). Physical properties of Moloney murine leukemia virus high molecular weight RNA: a two subunit model. J. Virol. 16, 1520-1535.

Rothenberg, E., Donoghue, D.J. and Baltimore, D. (1977). Analysis of a 5' leader sequence on murine leukemia virus 21S RNA: heteroduplex mapping with long reverse transcriptase products. Cell 13, 435-451.

Rungger, D. and Türler, H. (1978). DNAs of simian virus 40 and polyoma direct the synthesis of viral tumor antigens and capsid proteins in Xenopus oocytes. Proc. Natl. Acad. Sci. USA 75, 6073-6077.

Rutgers, T., Neeleman, L., van Vloten-Doting, L. Cleuter, Y., Hubert, E., Huez, G. and Marbaix, G. (1976). Translation products of the alf-alfa mosaic cell-free system and in oocytes from Xenopus laevis. Arch. Int. Physiol. Biochem. 84, 654-655.

Salden, M., Asselbergs, F.A.M. and Bloemendal, H. (1976a). Translation of oncogenic virus RNA in Xenopus laevis oocytes. Nature 259, 696-699.

Salden, M.H.L., Selten-Versteegen, A.-M. and Bloemendal, H. (1976b). Translation of Rauscher murine leukemia viral RNA. A model for the function of a virus-specific messenger. Biochem. Biophys. Res. Commun. 72, 610-618.

Schultz, A.M. and Orozlan, S. (1978). Murine leukemia virus gag polyproteins: The peptide chain unique to Pr80 is located at the N-terminus. Virology 91, 481-486.

Schwinghamer, N.W. and Symons, R.H. (1977). Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell-free systems and toad oocytes. Virology 79, 88-108.

Scupham, R.K., Jones, K.J., Sagik, B.P. and Bose, H.R. (1977). Virus directed post-translational cleavage in Sindbis virus infected cells. J. Virology 22, 568-571.

Semancik, J.S., Conejero, V. and Gerhardt, J. (1977). Citrus exocortis viroid: survey of protein synthesis in Xenopus laevis oocytes following addition of viroid RNA. Virology 80, 218-221.

Stacey, D.W. and Hanafusa, H. (1978). Nuclear conversion of microinjected avian leukosis virion RNA into an envelope glycoprotein messenger. Nature 273, 779-782.

Van Vloten-Doting, L., Bol. J., Neeleman, L., Rutgers, T., van Dalen, D., Castel, A., Bosch, H., Marbaix, G., Huez, G., Hubert, E. and Cleuter, Y. (1977). In vivo and in vitro translation of the RNAs of Alfalfa Mosaic Virus. In: Nucleic Acid and protein synthesis in plants (L. Borograd and J.H. Weil., eds.). pp. 387-411, Plenum. Publ. Co., New York.

Van Zaane, D. (1977). Thesis. Univ. of Nijmegen, Nijmegen, The Netherlands.

Van Zaane, D., Dekker-Michielsen, M.J.A. and Bloemers, H.P.J. (1976). Virus-specific precursor polypeptides in cells infected with Rauscher Leukemia virus: Synthesis, identification and processing. Virology 75, 113-129.

Van Zaane, D., Gielkens, A.J.L., Dekker-Michielsen, H.J.A. and Bloemers, H.P.J. (1975). Virus specific precursor polypeptides in cells infected with Rauscher Murine Leukemia Virus. Virology 67, 544-552.

Van Zaane, D., Gielkens, A.J.L., Hesselink, W.G. and Bloemers, H.P.J. (1977). Identification of Rauscher murine leukemia virus specific mRNAs for the synthesis of GAG and ENV gene products. Proc. Natl. Acad. Sci. USA 74, 1855-1859.

Von der Helm, K. (1977). Cleavage of Rous sarcoma viral polypeptide precursor to internal structural proteins in vitro involves viral protein p 15. Proc. Natl. Acad. Sci. USA 77, 911-915.

Yoshinaka, Y. and Luftig, R.B. (1977a). Properties of a proteolytic factor of murine leukemia viruses. Cell 12, 709-719.

Yoshinaka, Y. and Luftig, R.B. (1977b). Partial characterization of a p70 proteolytic factor that is present in purified virions of Rauscher murine leukemia virus. Biochem. Biophys. Res. Commun. 76, 54-63.

SAMENVATTING

Dit proefschrift beschrijft experimenten, waarin boodschapper-RNA (mRNA) ingespoten wordt in het cytoplasma van levende cellen, teneinde de vertaling van het mRNA te bestuderen onder omstandigheden, die zo dicht mogelijk de normale omstandigheden benaderen. Er werd gebruik gemaakt van de eicellen (oöcyten) van de Zuidafrikaanse klauwpad, Xenopus laevis, die door hun bijzondere grootte zeer geschikt zijn voor micro-injectie experimenten. Naast de experimenten van de auteur is een korte beschrijving van de biologisch-technische aspecten van dit soort experimenten met Xenopus eicellen opgenomen (hoofdstuk II) alsmede een uitgebreid overzicht van de literatuur over micro-injectie van mRNA (hoofdstukken III-1 en IV-1).

Bestudering van de regulatie van de vertaling van mRNA in eicellen vereist een methode om de aanmaak van de vertalingsproducten, de eiwitten, te meten. Drie methoden werden hiervoor gebruikt. (a) Door middel van gelelektroforese kunnen de (radioactief gemerkte) oöcyt-eiwitten worden gescheiden van de translatieproducten van sommige ingespoten mRNA's. Deze techniek blijkt geschikt om de vertaling van konijneglobine mRNA en van sommige kalfsooglens mRNA's te meten (hoofdstuk III-3). (b) Immuunprecipitatie van de verschillende soorten ooglens-eiwitten (crystallines) kan kwantitatief worden uitgevoerd door coprecipitatie met toegevoegde (niet radioactief gemerkte) gezuiverde ooglenseiwitten toe te passen (hoofdstuk III-2). (c) Immuunprecipitatie van de translatieproducten van het Rauscher muizeleukemie-virus (R-MuLV) kon gestandaardiseerd worden door met een ander radioactief isotoop gemerkte translatieproducten te gebruiken als interne standaard (hoofdstuk V-3). Met behulp van deze technieken werd

van het ingespoten mRNA maximaal is. De snelheid blijft dan in het algemeen constant gedurende enige dagen : ieder van de drie ingespoten soorten mRNA (globine-, crystalline- en RMuLV-mRNA), is stabiel in oöcyten. De efficiëntie, waarmee het ingespoten mRNA vertaald wordt, verschilt tussen groepen oöcyten, die geïsoleerd worden uit verschillende padden. Eicellen geïsoleerd uit eenzelfde pad vertalen crystalline mRNA's ongeveer tweemaal zo efficiënt als β-globine mRNA. De efficiëntie van de vertaling van RMuLV-RNA ligt in het algemeen ongeveer vijfmaal zo laag als die van β-globine-mRNA. De vertalingscapaciteit van de eicel is beperkt. Een boodschapper-RNA kan daarom verdrongen worden door een overmaat van een tweede mRNA. Of dit tweede mRNA meer of minder efficiënt vertaald wordt, speelt geen rol.(hoofdstuk III-3), Daarentegen ondervonden endogene en ingespoten mRNA's geen enkele competitie van "cap"-analogen, synthetische equivalenten van het 5'-uiteinde (de "cap") van mRNA's (hoofdstuk III-4). Deze resultaten tonen een aanzienlijk verschil aan tussen vertaling van mRNA in oöcyten en in celvrije systemen, want in celvrije vertalingssystemen wordt een efficiënt mRNA niet verdrongen door een overmaat van een inefficiënt mRNA en bovendien wordt de vertaling van mRNA <u>in vitro</u> sterk geremd door "cap"-analogen. In oöcyten blijken boodschapper-RNA's, die al vertaald worden, in het bijzonder de endogene mRNA's van de eicel, een voorsprong te hebben in de competitie met nog niet vertaalde mRNA's. Als de ingespoten mRNA's eenmaal met maximale snelheid vertaald worden (vanaf 6 a 8 uur na inspuiten) blijken ze ook beter bestand tegen competitie van pas ingespoten mRNA's en gedragen ze zich in dit opzicht als echte endogene mRNA's (hoofdstuk III-3).

Na de vertaling van de boodschapper-RNA's worden vaak nog bepaalde veranderingen aangebracht in de pas gemaakte eiwitketen. Wanneer <u>Xenopus</u>

eicellen worden ingespoten met een heteroloog (uit een ander type cel afkomstig) mRNA, worden deze post-synthetische modificaties van het translatieproduct meestal correct uitgevoerd (hoofdstuk IV-1). Twee voorbeelden hiervan zijn door de auteur bestudeerd : de assemblage van -crystalline (hoofdstuk IV-2) en de proteolytische splitsing van het primaire translatieproduct van retrovirus RNA, in het bijzonder van de RNA's van het vogelleukose-virus (AMV)(hoofdstuk V-2) en R-MuLV (hoofdstuk V-3). In Xenopus eicellen, maar niet in een celvrij systeem, worden de translatieproducten van de lens mRNA's tot -crystalline aggregaten bestaande uit ca. 40 subeenheden geassembleerd. De A-ketens alleen blijken ook een aggregaat te kunnen vormen. Waarschijnlijk wordt de aggregatie door het A-crystalline geïnitieerd, waarna de B-ketens opgenomen worden in het aggregaat. De post-synthetische splitsing van het translatieproduct van het retrovirus RNA in de vier structurele eiwitten van het viruskerntje verloopt ook niet in vitro, maar wel in eicellen. De splitsingen verlopen langzamer in oöcyten dan in de door de virussen geïnfecteerde cellen van warmbloedige dieren. Daardoor kunnen de splitsingsintermediairen beter bestudeerd worden en kan de volgorde van de virale eiwitten in het 'polyproteine' gemakkelijker bepaald worden.

CURRICULUM VITAE

Fred A.M. Asselbergs was born September 26, 1949 at Bergen op Zoom, The Netherlands. In 1968 he graduated from the "Dr. Mollerlyceum" at Bergen op Zoom. In September of the same year he started his biology studies at the State University of Utrecht, Utrecht, The Netherlands. He obtained his bachelor's degree in June 1971,In December 1974 he obtained his master's degree majoring in biophysical chemistry, with fysiological chemistry and experimental tumorimmunology as minor subjects. For his scientific research work on Semliki Forest Virus done during his studies he obtained the 1974 Faculty prize.

In November 1974 he joined the Department of Biochemistry of the University of Nijmegen, Nijmegen, The Netherlands (head: Prof. H. Bloemendal) to work on translation of eye lens mRNA in cell-free systems and in Xenopus oocytes. Soon he became also involved in the department's research on translation of leukemia virus mRNA's. These investigations were partly carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid of the Netherlands Organisation for the Advancement of Pure Research (ZWO).

In Januari 1979 he obtained a post-doctoral fellowship from the Robertson Research Fund to work at the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. He is presently studying the regulation of gene expression of human adenovirus using the Xenopus oocyte as a coupled transcription-translation system.

# STELLINGEN

## I

Wanneer "in vitro"-translatie gebruikt wordt om de rol van de faktoren bij de initiatie van de eiwitsynthese te bestuderen, is het essentieel onderscheid te maken tussen de eerste initiatie-reaktie waarin een bepaald boodschapper RNA betrokken is, en daaropvolgende initiaties op hetzelfde RNA molecuul

*Asselbergs, F A.M., Peters, W , Van Venrooij, W J , en Bloemendal H. (1978). Eur. J Biochem. 88, 483-488*

## II

De twee polyproteinen pr76 en pr65 waarvoor het *gag*-gen van het muizeleukemie virus codeert, zijn vertalingsprodukten van twee verschillende boodschapper RNA's

*Phillipson, L., Anderson, P , Olshevsky, U , Weinberg, R , Baltimore, D , en Gesteland, R (1978) Cell 13, 189-199*
*Edwards, S A , en Fan, H (1979) J Virol 30, 551-563*
*Dit proefschrift*

## III

De intracellulaire interferonconcentratie is geen goede maatstaf om het verloop van de vertaling van het interferon-mRNA te bestuderen De conclusie van Seghal en medewerkers, dat het poly-(A)-segment van dit mRNA niet nodig is voor de stabilisatie ervan in *Xenopus* eicellen dient derhalve in twijfel te worden getrokkén

*Seghal, P B , Soreg, H en Tamm, I (1978) Proc Natl Acad Sci. USA 75, 1614-1618*

## IV

Het gebruik door Van der Donk en zijn medewerkers van de term "precursor" voor een niet-covalent gebonden complex van calcitonine met een ander eiwit is niet correct

*Van der Donk, J A , Van Dam, R.H., Goudswaard, J , Hackeng, W.H L en Lips, C.J M (1976). Lancet II, 1133*

V

Bij het gebruik van celvrije vertaling als zuiverheidscriterium voor histon boodschapper-RNA's, hebben Liautard en Jeanteur onvoldoende rekening gehouden met de afwijkende aminozuursamenstelling van de histon-eiwitten

*Liautard, J.P en Jeanteur, Ph (1979) Nucleic Acids Res 7, 135-150*

VI

Chatterjee en zijn medewerkers noemen de boodschapper-RNA-bevattende deeltjes die zij uit de celkern isoleren ten onrechte "polyribosomen"

*Chatterjee, N K, Dickerman, H W en Beach, T A (1977) Arch. Biochem Biophys 183, 228-241*
*Chatterjee, N K, Sarma, M H, Dickerman, H W en Beach, T A (1979) J Biol Chem. 254, 6156-6162*

VII

De uit het Engels afkomstige benaming van de gras-etende breedlipneushoorn (*Ceratotherium simum*), de zogenaamde witte neushoorn, is naar alle waarschijnlijkheid afkomstig van het Zuidafrikaanse woord "weide" en niet van "wijde" zoals vaak verondersteld wordt

*B Grzimek ed, Animal Life Encyclopedia, Vol 13*

VIII

Zolang het type en de gevoeligheid van rolfilms niet aangegeven staan op het dekseltje van het filmdoosje in plaats van nu op de zijkant, blijft het "handige" informatieraampje op de achterkant van veel camera's van informatie verstoken

Nijmegen, 8 november 1979 F A M Asselbergs